The

Amino Acid Composition

of

Proteins and Foods

ANALYTICAL METHODS AND RESULTS

PROFESSOR AND MRS. LAFAYETTE B. MENDEL AT HOME,
DELHI, NEW YORK *ca.* 1932

The

Amino Acid Composition

of

Proteins and Foods

ANALYTICAL METHODS AND RESULTS

Second Printing

By

RICHARD J. BLOCK, Ph.D.

Associate in the Department of Physiology and Biochemistry, New York Medical College; Research Director, C. M. Armstrong, Inc., New York City; Formerly Research Associate in Chemistry, New York State Psychiatric Institute and Hospital, New York City; Consultant in the Chemistry of Proteins and Amino Acids.

and

DIANA BOLLING, B.S.

PREFACE

THE current trend of the investigation of the chemistry of nutrition is emphasizing the significance of the amino acids as the fundamental factors in all problems in which hitherto the rôle of proteins has been involved. . . . Obviously the relative values of the different proteins in nutrition are based upon their content of these special amino acids which cannot be synthesized in the animal body and which are indispensable . . ." These views, set forth by T. B. Osborne and L. B. Mendel in 1914, cover the accepted facts on protein nutrition today. The extension of the pioneer experiments of Willcock and Hopkins, Osborne and Mendel, and others were brought to a successful conclusion by the well known results of one of Professor Mendel's distinguished students, Professor W. C. Rose of Illinois.

With the proof of the essential nature for animal nutrition of methionine, histidine, lysine, tryptophane, phenylalanine, threonine, leucine, isoleucine, and valine, and the special importance of cystine, arginine, tyrosine, and glycine as shown by W. C. Rose, H. J. Almquist, R. W. Jackson, H. H. Mitchell, and others, the nutritive evaluation of protein foods, based on their comparative amino acid composition, became a possibility. A reasonably accurate knowledge of the amino acid composition of a protein permits an approximation of its nutritive value and, more important, allows the choosing of different proteins so that they become mutually supplementary. The formulation of diets based on amino acid composition results in a great saving of time and cost over the long and tedious trial and error type of animal test employed heretofore.

W. E. Gaunt (255) has said recently (1942): "Supplies of protein foods for both the human and the stock populations of Great Britain are limited, and this limitation will continue after the War. For the most economical use of these supplies, it is obviously of the utmost importance for us to determine as rapidly as possible the qualitative and quantitative amino acid requirements of farm animals and man." If the amino acid requirements of an animal are known, even approximately, the proper quantities and combinations of food proteins can be chosen, provided that their essential amino acid composition has been estimated by methods of comparable accuracy (cf. Chapter XI). Evidence is beginning to accumulate concerning the special importance of certain amino acids in pre- and post-operative treatment, in wound healing, in blood regeneration, in learning processes, and even in the etiology

v

of mental disease. Again, a knowledge of the amino acids present in available proteins may permit the use of relatively inexpensive sources rather than the more costly purified amino acids. Amino acid analysis has revealed rich sources of specific amino acids in protein products which were heretofore unsuspected. Protein analysis is also valuable in revealing new uses of the protein containing by-products of industry, and, in quickly, revealing specific deficiencies in natural and prepared foodstuffs.

The degree of experimental accuracy in the amino acid analysis of the naturally occurring heterogeneous proteins, which may vary within wide limits because of preparative difficulties as well as changes in the amino acid composition of the tissue or organism itself, does not need to be as high as that required in a study of the molecular structure of those rare proteins which have been shown to be homogeneous substances. Although, in all analytical work, one must strive for the determination of absolute values, nevertheless, much valuable information of a permanent nature can be achieved by comparative amino acid analyses, especially in relation to protein nutrition.

This monograph gives many of the analytical values from the literature as well as some hitherto unpublished experiments of our own. Those proteins, for which only a few analytical values of questionable accuracy are available, have been omitted; as have also the majority of those analyses on purified plant proteins which represent only a small proportion of the total proteins of the plant.

Although the number of foodstuffs which have been analyzed, by even reasonably accurate methods, is very limited, it is the purpose of this monograph to summarize the data which have come to the authors' attention, in such a fashion that the analytical values will become more easily accessible and widely available. In order to permit the reader to evaluate the data more accurately, nearly all of the analytical procedures which have been employed by various investigators are presented in detail. Although this may lead to a certain amount of repetition, especially in the first three chapters, it is hoped that the advantages outweigh the disadvantages.

The methods described include procedures for the estimation of those essential and non-essential amino acids which can be evaluated with some degree of accuracy. The analytical procedures are arranged along the lines which were forged by the methods themselves, for example, the basic amino acids fall into one group: tyrosine, tryptophane and phenylalanine form another; the dicarboxylic amino acids a third; serine and threonine form a fourth; etc.

We are greatly indebted to Dr. W. D. Block, University of Michigan; Dr. M. John Boyd, University of Cincinnati; Dr. W. L. Brown, Georgia Experiment Station; Dr. J. W. Cavett, Charles City, Iowa; Professor A. C. Chibnall, London, England; Dr. Theodore F. Lavine, Lankenau Hospital; Dr. W. R. Murrill, University of Michigan; Dr. Ben H. Nicolet, United States Department of Agriculture; Dr. F. E. Reinhart of the Franklin Institute; and Dr. M. X. Sullivan of Georgetown University for the use of analytical procedures and results prior to publication. We also wish to thank Mr. J. R. Bishop, Chief, Cereal Grain Division, War Production Board; Drs. A. P. Hellwig, B. F. Buchanan, and H. H. Schopmeyer, the American Maize-Products Co.; Dr. G. C. Supplee, the Borden Co.; Dr. H. G. Dunham, Difco Laboratories; Dr. B. F. Oser, the Food Research Laboratories; Dr. C. N. Frey, Standard Brands, Inc.; and Mr. Arthur Wendel, President, the Wheatena Corporation—for permission to include many analytical data on foods, food products, and special protein hydrolysates.

Many of the thoughts and ideas expressed in this monograph are the result of the kind suggestions and criticisms of various scientists, including, Drs. Max Bergmann, R. Keith Cannan, George R. Cowgill, Icie Macy-Hoobler, Nolan D. C. Lewis, William A. Perlzweig, Carl L. A. Schmidt, M. X. Sullivan, Donald D. Van Slyke, Hubert B. Vickery and, above all, the late Professor Lafayette B. Mendel.

We are especially indebted to Mrs. Paul Kerner for her meticulous stenographic work and for her preparation of the indices; and to Mr. Merrill Webb, U.S.N. for his invaluable aid in the preparation of the bibliography.

PREFACE TO SECOND PRINTING

In the second printing of this monograph, it was not possible because of printing difficulties to attempt to correct the errors and omissions of the earlier printing which were brought to our attention both by private correspondence and in carefully considered reviews. However, the most severe criticism, mentioned only once, concerned the mode of presenting the analytical data. Although we believe that the method of presentation in the earlier printing (a method which did not originate with us) was the most suitable for the majority of users of this monograph, the fact that values could be interpreted in a way never intended by the authors, has caused us to change the form of the tables in this printing. The scheme used in this present printing was arrived at after consulting with a number of eminent protein chemists and nutritionists.

Our object in publishing this monograph is to present to the average reader the widely scattered literature on the methods and results of protein analysis in the most easily useable form. We have no pet ideas to propound and continue to appreciate receiving suggestions for its future improvement.

INTRODUCTION [e]

IN PREPARING a monograph on the methods and results of amino acid determinations, it is proper to dwell briefly on the object and limitations of such work.

If a study of the structure of a pure homogeneous protein is the point at issue, then determinations of a few amino acids with the highest possible accuracy is of infinitely greater value than rough estimations of the approximate quantity of a large number. Thus, for purely chemical and physico-chemical studies, accurate estimations are prerequisite; while for a broad survey of the field of nutritionally valuable food proteins, the first need is to gain as wide a knowledge as possible of the qualitative and quantitative distribution of the essential amino acids. It has been our experience that a food protein may be a good source of those nutritionally valuable amino acids which are most commonly estimated (i.e., cystine, methionine, arginine, histidine, lysine, tyrosine, and tryptophane) and yet be deficient in one or more of the other essentials for which analytical methods are more difficult and, often, less accurate. If the analytical results of only the first group of amino acids were the sole basis of evaluating the protein, a serious error *may have been made in estimating the biological quality of the* protein. Thus, the analysis of *all* the amino acids of special nutritional importance, even though some of the results must, at present, be of comparative rather than absolute significance, will yield valuable and pertinent information.

Wherever possible, several procedures have been given for the estimation of each amino acid. If the identical analytical result is obtained by two entirely different procedures, a degree of confidence is imparted to the values which is not given even by closely checking replicate estimations by the same method. It is to be regretted that only a few investigators have used two or more methods for the determination of a single amino acid in the same preparation. It is hoped that the summarizing in one place of the many methods which have been offered for the estimation and determination of amino acids will encourage this type of study.

There is, however, one stumbling block in the accurate determination of amino acids in proteins which must be constantly reemphasized. Amino acids methods, with few exceptions, require hydrolysis of the protein before they can be applied. The susceptibility of each and every amino acid to loss during hydrolysis is different and differs not only with the conditions of hydrolysis, but

more so with the presence or absence of other substances in the hydrolysis mixture. Thus, various investigators have shown that cystine and especially cysteine may be destroyed during acid hydrolysis when carbohydrates are present, but not in their absence.

Attempts have been made to evaluate hydrolytic losses by adding the amino acid under investigation both before and after hydrolysis. The apparent destruction during hydrolysis can then be determined by the difference in the analytical results obtained. This requires the tacit assumption that the amino acid added to the protein reacts during the hydrolysis in exactly the same way as its analogue in the peptide linkage. A fact which is known to be untrue in the case of certain amino acids. However, this procedure is, it is believed, the best approximation that can be made at the present time whenever hydrolysis is required.

It is interesting to note that in a considerable proportion of the special procedures for protein analysis which have been applied without preliminary hydrolysis, the analytical values obtained to date have been equal or even lower than those following hydrolytic fission.

As in the case of all branches of science, the great majority of methods which have been used, are modifications of earlier procedures. It will be the policy of the writers, wherever possible to refer to a method first by the name of the investigator who initially developed or applied it to amino acids; and secondly wherever feasible, by the name of the modification used in the specific investigation. Thus, the Vickery and Leavenworth (644, 645) modification of the Kossel-Kutscher (379) procedure for the separation and isolation of the basic amino acids will be referred to as the Kossel-Vickery method. This system of nomenclature is not uncommon in analytical chemistry and facilitates recalling the general procedure while indicating the particular improvement followed.

In the interest of uniformity and to facilitate comparison, the analytical figures given in this monograph have been calculated to 16.0 grams of nitrogen, wherever possible. In certain cases where nitrogen values were not given but the determinations were calculated on the basis of the moisture, fat, and ash-free preparation the data have been recalculated to 16.0 grams of nitrogen using a generally accepted value for N which is always indicated by placing the N value in parenthesis. It is recognized that this procedure may introduce a certain error, but it is believed more suitable than other methods of presentation.

As all the data in the tables are calculated on the basis of 16 grams

of nitrogen, it is only necessary to know the nitrogen content of the protein in order to recalculate the data in the tables to give the approximate amino acid composition of the preparation. If the protein contains 18.6 per cent of nitrogen on a moisture and ash-free basis, then the values in the proper table are multiplied by the factor

$$\frac{18.6}{16.0} \text{ or } 1.16$$

If the nitrogen of the product is only 12.2 per cent, then the amino acid figures are multiplied by the factor

$$\frac{12.2}{16.0} \text{ or } 0.76$$

Although a complete survey of the literature on protein analyses has not been attempted, the authors realize that many valuable contributions, especially those in foreign journals and in periodicals not devoted primarily to physiological chemistry, have been unintentionally omitted. It is also probable that, due to the many calculations and transpositions of data from the literature, which were necessary in the preparation of this monograph, numerical errors have been made. The authors welcome the correction of any erroneous statements or calculations and hope that readers will be so kind as to call their attention to important papers on this subject that have been omitted.

We wish to acknowledge our indebtedness to the writers and publishers of the various scientific books and journals from which the greatest part of the descriptions of experimental details, the analytical results, and almost all the figures and diagrams were taken. Although specific credit is given in the text or bibliography, it is hoped that any omissions will be brought to the authors' attention for rectification.

CONTENTS

The

Amino Acid Composition

of

Proteins and Foods

ANALYTICAL METHODS AND RESULTS

CHAPTER I

THE DIAMINO ACIDS

ARGININE, HISTIDINE, LYSINE, HYDROXYLYSINE, AND CITRULLINE

Amino Acid	Formula	Optical Form	Molecular Weight	Percentage Composition			
				C	H	N	O
Arginine	$C_6H_{14}O_2N_4$	d	174.14	41.35	8.10	32.18	18.37
Histidine	$C_6H_9O_2N_3$	l	155.09	46.42	5.85	27.10	20.63
Lysine	$C_6H_{14}O_2N_2$	d	146.13	49.27	9.66	19.17	21.90
Hydroxylysine	$C_6H_{14}O_3N_2$		162.13	44.08	8.70	17.28	29.61
Citrulline	$C_6H_{13}O_3N_3$		175.12	41.11	7.48	23.99	27.41

PART I

1. THE ISOLATION AND SEPARATION OF THE BASIC AMINO ACIDS ACCORDING TO KOSSEL (378, 379)

HISTORICAL

IN 1898, Kossel (378) reported the isolation and separation of the basic amino acids from protamines. Histidine was precipitated from the hydrolysate with $HgCl_2$ from a neutral or weakly alkaline solution and isolated as the hydrochloride. Arginine was precipitated in the presence of excess silver ions ($Ag_2SO_4 + AgNO_3$) by barium hydroxide in strongly alkaline solution and isolated as the nitrate. Lysine was precipitated, after removal of the histidine and arginine, by phosphotungstic acid in dilute sulfuric acid. The lysine was isolated as the picrate.

As will be seen below with the exception that more convenient salts have been found for the isolation of histidine and of arginine, the Kossel method has been improved in detail only.

EXPERIMENTAL*

A. The Procedure of Kossel and Kutscher (379)

Principle: The protein is hydrolyzed with a strong mineral acid and after removal of the excess acid, arginine and histidine are precipitated *together as their silver salts in strongly alkaline solution*. These bases can be separated because silver precipitates

* Recommended procedures are starred.

3

histidine quantitatively at neutral reaction while arginine does not come down until the silver salts are alkalinized with $Ba(OH)_2$. Lysine is separated from the first filtrate by precipitation with phosphotungstic acid.

Histidine is isolated as the dihydrochloride. Arginine is isolated as the nitrate, and lysine is obtained as the picrate. A correction (0.036 gm. of arginine per liter) for the solubility of arginine silver in the presence of excess $Ba(OH)_2$ is applied (Gulewitsch, 270).

Reagents: Commercial phospho-24-tungstic acid is purified (Winterstein's method, 686) by shaking a dilute hydrochloric acid solution of the same with ether. The ether layer which contains the desired product, is separated and removed. The phosphotungstic acid is crystallized from water.

Method: 1. Hydrolysis. 25–50 gm. of protein are hydrolyzed under reflux for 8–14 hrs. with 10 volumes of *one* of the following acid mixtures:

   a. 150 gm. of concentrated $H_2SO_4 + 300$ ml. of H_2O.

   b. 162 ml. of concentrated $H_2SO_4 + 324$ ml. of H_2O.

   c. 114 gm. $I_2 + 14$ gm. amorphous $P + 86$ ml. of H_2O, reflux to dissolve and use the HI as prepared.

2. Removal of Excess Acid and Humin. The solution is cooled, water is added, and then the excess acid is removed with barium hydroxide in the case of H_2SO_4 and with silver nitrate and silver sulfate in the case of HI. The precipitate is washed repeatedly with hot water.

3. Removal of NH_3. The amino acid solution is concentrated to approximately 1 liter and an excess of magnesia is added. The alkaline suspension is warmed to drive off the ammonia.

4. Precipitation of Histidine and Arginine. After removal of the magnesia, the solution (vol. $= 3$ L.) is treated with an excess of Ag_2SO_4 at 40°. Excess silver ion is tested for by adding a drop of the filtrate to a few ml. of cold saturated $Ba(OH)_2$. A brown precipitate indicates an excess of Ag^+. This is the important brown spot test still in use. The solution is now saturated with powdered $Ba(OH)_2 \cdot 8 H_2O$. The resulting precipitate of arginine and histidine silver is washed with cold saturated $Ba(OH)_2$. The filtrate is set aside as it contains *lysine*.

5. Separation and Isolation of Histidine. The arginine and histidine silver salts are decomposed in dilute H_2SO_4 by H_2S. After the Ag_2S and H_2S have been removed, an excess of $AgNO_3$ is added and the histidine is precipitated by the careful addition of cold saturated $Ba(OH)_2$ to neutrality and then to the point where ammoniacal $AgNO_3$ no longer gives a precipitate when added to the

histidine silver filtrate. The precipitate of histidine silver is washed with water. The filtrate contains *arginine*.

The histidine silver precipitate is decomposed with dilute H_2SO_4 and H_2S. After removal of the Ag_2S and H_2S, the H_2SO_4 is removed with $Ba(OH)_2$ and the latter with CO_2. The $BaSO_4$ and $BaCO_3$ are filtered off, thoroughly washed with hot water, and the filtrate is concentrated to a syrup. The syrup is extracted with 10–20 per cent $AgNO_3$ containing 1 drop of dilute HNO_3. Any precipitate is discarded. The histidine is again precipitated as the silver salt with NH_4OH. The histidine silver precipitate is removed and decomposed with hot dilute HCl. The clear filtrate from the AgCl is concentrated to a small volume and *histidine dihydrochloride* is removed and weighed; $C_6H_9N_3O_2 \cdot 2$ HCl after drying at 40° *in vacuo*.

6. Separation and Isolation of Arginine. The filtrate from the histidine silver separation is saturated with powdered $Ba(OH)_2$. The arginine silver precipitate is washed nitrate-free with cold saturated $Ba(OH)_2$ and then it is decomposed with H_2SO_4 and H_2S. The precipitate of $BaSO_4$ and Ag_2S is thoroughly washed with hot water. The excess H_2SO_4 is removed with $Ba(OH)_2$ and the latter is taken out of solution with CO_2. The alkaline filtrate of arginine carbonate is neutralized with HNO_3 and the solution is concentrated *in vacuo* to dryness. The residue is weighed as arginine nitrate, $C_6H_{14}N_4O_2 \cdot HNO_3 \cdot \frac{1}{2}H_2O$.

A correction of 0.036 gm. of arginine per liter of $AgNO_3$-$Ba(OH)_2$ solution is applied (270).

7. Isolation of Lysine. The filtrate from the initial separation of arginine and histidine is acidified with H_2SO_4 and the Ag^+ is removed with H_2S. The precipitate of $BaSO_4$ and Ag_2S is thoroughly washed with hot water as usual, and the solution is concentrated to 500 ml. Sufficient H_2SO_4 is added to make a final concentration of 5 per cent by weight. A 20 per cent solution of purified phospho-24-tungstic acid in 5 per cent H_2SO_4 is added to the lysine fraction until there is no further precipitate for 10 seconds after adding the reagent. The lysine phosphotungstate, after standing at room temperature over night, is removed by filtration and carefully washed with 5 per cent H_2SO_4. The lysine precipitate is decomposed with an excess of $Ba(OH)_2$. The barium phosphotungstate is thoroughly washed with dilute $Ba(OH)_2$ and the Ba^{++} is removed with CO_2. The $BaCO_3$ is washed with hot water and the filtrate is concentrated to a small volume. A solution of picric acid in absolute alcohol is added in small portions as long as a precipitate appears. The lysine picrate is removed and recrystallized from a small volume of water. Lawrow (409) found that the solubility of lysine

picrate in water at 21–22° was 0.54 gm. per 100 ml. However, even when this correction is applied the results of the recrystallized product remain low. Therefore, it is preferable to decompose the crude lysine picrate with dilute H_2SO_4, remove the picric acid with ether, and to reprecipitate the lysine as the phosphotungstate. Tyrosine remains in the lysine phosphotungstate mother liquors. The lysine phosphotungstate is decomposed as above and the base is isolated as the pure lysine picrate.

Comment: Kossel and Kutscher used Ag_2SO_4 in the initial precipitation in order to avoid the effects of HNO_3 on the lysine. The disadvantage of Ag_2SO_4 is that it is relatively insoluble even at 40° and therefore they advise that if a positive brown spot test cannot be obtained at a volume of 3 liters, the hydrolysate should be further diluted.

These investigators recognized that the isolation method gave *minimal* results, but they stressed its comparative value especially when all conditions, i.e., the volumes of the arginine silver and lysine phosphotungstate filtrates, were kept constant.

B. Kossel and Patten's Modification (380)

Principle: Histidine is quantitatively precipitated by $HgSO_4$ in dilute H_2SO_4. This step is used to remove aspartic acid, etc. which may be precipitated to a certain extent by silver at neutrality.

Reagents: 75 gm. of HgO are dissolved by warming with 500 ml. of 15 per cent (by volume) of H_2SO_4.

Method: The procedure is essentially the same as that of Kossel and Kutscher (379) except that $BaCO_3$ is used in place of magnesia to remove the humin and NH_3.

After the histidine silver has been precipitated at neutrality and the inorganic reagents have been removed, the solution is concentrated so that it contains $2\frac{1}{2}$ per cent of H_2SO_4 and the histidine is precipitated by the addition of a slight excess of $HgSO_4$ in H_2SO_4 solution. The mixture is allowed to stand over night. The histidine mercury precipitate is removed, washed with dilute $HgSO_4$ reagent and decomposed with H_2S. The HgS is filtered off and thoroughly washed with hot water. The H_2S is then removed from the filtrate by boiling and the H_2SO_4 is precipitated with $Ba(OH)_2$. The latter is removed with CO_2. The alkaline histidine solution is acidified with HCl and histidine dihydrochloride is obtained by concentration to a small volume in a porcelain evaporating dish.

Comment: It was recognized that tryptophane and cystine are also precipitated by $HgSO_4$, but these amino acids can easily be tested for by the nitroprusside and Hopkins-Cole reactions.

Although most investigators using the Kossel procedure were accustomed to isolate both arginine and histidine salts for the purpose of identification, many of the analytical values reported in the literature for these two amino acids were calculated from the total nitrogen of the final purified fractions. The nitrogen results were usually 10–15 per cent higher in the case of arginine and 20–30 per cent higher in the case of histidine than when calculated on the weight of the purified salts (494, 422, 643, and others).

C. Kossel and Pringle Modification (381)

Principle: Arginine and histidine silver salts can be separated by adding an excess of $BaCO_3$ at water bath temperature.

Comment: These investigators showed that histidine is quantitatively precipitated at a faintly alkaline reaction by silver, using Pauly's diazo reaction (513) to follow the completeness of the reaction. This was confirmed by Kossel and Staudt (385 and others).

D. Kossel and Staudt's Method (385)

Principle: Arginine and histidine are precipitated together as the silver salts at pH 13–14. After removal of the reagents, the arginine is quantitatively precipitated as the flavianate (Kossel and Gross, 384) and the histidine is calculated as the difference between total N and N precipitated with flavianic acid.

Comment: This procedure is useful in the analysis of protamines and other simple mixtures of amino acids, but is not advised when complex protein hydrolysates are to be investigated as the results for histidine will seldom be significant.

F. The Modification of Osborne, Leavenworth, and Brautlecht (494)

Principle: Arginine and histidine are precipitated together by $AgNO_3$-$Ba(OH)_2$ instead of Ag_2SO_4-$Ba(OH)_2$ to keep down the volume of solution. Histidine is separated from arginine by $HgSO_4$ in 5 per cent H_2SO_4. This method removes most of the histidine and the final amount is precipitated by $AgNO_3$-$Ba(OH)_2$ to neutral to litmus followed by the careful addition of $Ba(OH)_2$ until 1 drop of cold saturated $Ba(OH)_2$ to 10 ml. of clear filtrate gives no precipitate.

Comment: A study of the purity of the final amino acid fractions gave the following results. Arginine copper nitrate accounts for 85–90 per cent of the nitrogen of the arginine fraction. Histidine dihydrochloride accounts for 75–80 per cent of the N of the histidine fraction. Lysine must not be calculated from N, but only from the weight of the picrate.

Good determinations can be obtained only after considerable experience.

The length of hydrolysis was increased from 12 to 24 hours.

G. Vickery and Leavenworth's 1927 Modification (642)

Principle: Histidine is completely precipitated by silver and barium·hydroxide at a pH above 6.6. Arginine does not begin to come down until pH 7.9. The histidine silver precipitate may however include a small quantity of arginine which is best removed by decomposing the silver precipitate with hot dilute HCl and reprecipitating with silver at pH 7.0.

Arginine is precipitated by silver and barium at pH 10–11. It has been claimed that it is not necessary to saturate the solution with $Ba(OH)_2$ as suggested by Kossel (379) and that if the precipitation is carried out near pH 11, the Gulewitsch (270) correction for the solubility of arginine silver need not be used.

Comment: Bussit (137 found that histidine is not completely precipitated until the pH is 7.0 to 7.2. Later experiments showed that arginine, too, is often not quantitatively precipitated below pH 13–14 and that the Gulewitsch factor gives added validity to the results obtained by the Kossel silver method.

H. Vickery and Leavenworth's 1928 Modification (643)

Principle: Osborne's (494) use of $AgNO_3$ is avoided and replaced with Ag_2O plus H_2SO_4, according to Kiesel (361). Arginine is isolated as the flavianate (Kossel and Gross, 384). Histidine is isolated as the diflavianate.

Reagents: Moist Ag_2O is prepared from $AgNO_3$ and NaOH. The suspension is washed with water until nitrate free.

Method: 1. Hydrolysis. 50 gm. of protein (edestin) were hydrolyzed with 500 ml. of 20 per cent HCl for 30 hrs. i.e. until the ratio of total N: amino N became maximum. The excess acid was removed by repeated concentration in vacuo. The solution was diluted to 2 liters, H_2SO_4 was introduced from time to time to keep the reaction strongly acid while a suspension of moist Ag_2O was added until Kossel's brown spot test became positive. The precipitate of AgCl was removed by centrifuging and thoroughly washed with hot water. The filtrate was concentrated to 2 L. *in vacuo.*

2. Precipitation of Arginine and Histidine. The solution was tested for excess silver, if positive, hot saturated $Ba(OH)_2$ was added to alkaline to phenolphthalein. The silver salts were removed and washed once with dilute $Ba(OH)_2$. The filtrate and washings were set aside for *lysine.* The precipitate was decomposed with

H_2SO_4 and H_2S and the resulting filtrate was concentrated to 2 L.

3. Separation of Arginine and Histidine. Excess Ag_2O was added, care being taken that the reaction remained *acid* to Congo red paper. Then cold saturated $Ba(OH)_2$ was added to pH 7.0—bromthymol blue was used as the external indicator. The histidine silver precipitate was removed. The filtrate contained *arginine*.

4. Isolation of Histidine. The precipitate was decomposed with hot dilute HCl. The AgCl was washed with hot water. The filtrate was concentrated to dryness and the excess HCl was removed with $Ag_2O–H_2SO_4$. The silver was taken out with H_2S and the latter was removed by boiling. The solution was concentrated to 300 ml., H_2SO_4 was added to 5 per cent by weight and an excess of 10 per cent $HgSO_4$ in 10 per cent H_2SO_4 was added. The precipitate was allowed to form for 48 hours after which it was removed and washed with dilute reagent. The histidine mercury precipitate was then decomposed with H_2S. The reagents were removed, only a small amount of H_2SO_4 should remain in the solution at this point. The solution was brought to volume and its nitrogen content was determined. The remainder was then concentrated to 40–50 ml. and an excess of flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid) was added to the hot solution. Each gm. of histidine N requires 14.96 gm. of dye. The flavianate was crystallized in two crops and the precipitate was washed with alcohol and ether.

$$\text{Histidine} = 0.1979 \times \text{weight of histidine diflavianate}$$
$$\text{M.P.} = 251° \text{ when pure}$$
$$\text{M.P.} = 230° \text{ when crude}$$

5. Isolation of Arginine. The filtrates from the two histidine precipitations were weakly acidified with H_2SO_4 and were concentrated to 2 liters. Hot saturated $Ba(OH)_2$ was then added to alkaline to phenolphthalein and the precipitate of arginine silver was centrifuged and washed with dilute baryta. The precipitate was then decomposed with H_2SO_4 and H_2S and the arginine sulfate solution was concentrated to 500 ml. An aliquot of this solution was removed for the determination of nitrogen. 1 gm. of arginine N *requires 5.61 gm. of flavianic acid. A slight excess of reagent should be added to the arginine solution at boiling temperature.* Arginine flavianate precipitated as glistening orange plates while the solution was still warm. The precipitate was washed with water, alcohol, and ether and dried at 110° to constant weight.

$$\text{Arginine} = 0.3566 \times \text{weight of arginine flavianate.}$$

6. Separation and Isolation of Lysine. The filtrates from the alkaline silver precipitations (sections 2 and 5 above) were strongly

acidified with H_2SO_4 and the silver was removed with H_2S. The solution was concentrated, made alkaline to phenolphthalein with $Ba(OH)_2$ and the ammonia was removed by concentration *in vacuo*. The reagents were removed and the filtrate was concentrated to 500 ml. H_2SO_4 was added until its concentration equaled 5 per cent by weight. An excess of 20 per cent phospho-24-tungstic acid in 5 per cent H_2SO_4 was added and the precipitate was allowed to form over night. The precipitate was removed and washed twice with 2 per cent phosphotungstic acid in 5 per cent sulfuric acid. The lysine phosphotungstate was dissolved in acetone, water was added, and the lysine phosphotungstate was decomposed with an excess of hot saturated baryta. The barium phosphotungstate was washed twice with cold dilute $Ba(OH)_2$. The filtrates were collected and the barium was removed exactly with H_2SO_4. The filtrate must be free of both Ba^{++} and SO_4^{--} ions. The $BaSO_4$ was washed thoroughly with hot water as usual. The lysine carbonate solution was concentrated to 500 ml. Nitrogen was determined on an aliquot. The remainder of the solution was concentrated to a thin syrup, absolute alcohol was added to a faint turbidity, and slightly less than the calculated quantity of picric acid in absolute alcohol was added. The lysine picrate was allowed to crystallize out over night. The precipitate was filtered off, washed with cold absolute alcohol and ether and dried at 110°. If the M.P. of the lysine picrate was 250°, it was considered sufficiently pure, otherwise it was recrystallized from hot water, using Lawrow's (409) factor for the solubility of lysine picrate in water of 0.54 gm. per 100 ml.

$$\text{Lysine} = 0.3895 \times \text{weight of lysine picrate.}$$

Comment: The importance of washing all insoluble inorganic precipitates such as $BaSO_4$, $BaCO_3$, Ag_2S, HgS, etc. very thoroughly with hot water and the desirability of carrying out all concentrations at a low temperature *in vacuo* cannot be stressed too often.

The absolute accuracy of the analytical results can be further improved by working over all filtrates after removing the reagents.

Vickery and Leavenworth (643) report that 90 per cent of both histidine and arginine were recovered by the above procedure. Approximately 80–90 per cent of the nitrogen in the histidine fraction, 90 per cent of the nitrogen of the arginine fraction, and 60 per cent of the nitrogen of the lysine fraction could be isolated as the pure compounds. In confirmation of Kossel and Patten (380), these investigators found dicarboxylic amino acids in the histidine fraction.

Vickery and Leavenworth (645) reported in a later paper that difficulty was often encountered in obtaining a positive test for excess silver ions in solution by the Ag_2O-H_2SO_4 method even when

Ag_2SO_4 was crystallizing out of solution. This is a serious disadvantage to the Ag_2SO_4 method.

They, also, suggested raising the pH at which histidine is precipitated from 7.0 to 7.4 and then working up the $HgSO_4$ filtrate to recover any arginine which might be carried down in the histidine silver precipitation. Vickery and Leavenworth (645) believe that the small quantity of histidine, which they found in the arginine fraction, was due to the solubility of histidine silver. This is contrary to the experience of most observers who find that the precipitation of histidine by silver can be made quantitative (101, 137, 380, 385, 642, 23).

I. Vickery and Leavenworth's 1929 Modification (647)

Principle: The histidine fraction is freed of cystine by precipitating the latter with copper hydroxide.

Method: After the histidine mercury precipitate has been decomposed with H_2S and the excess reagents have been removed, the reaction is adjusted to pH 7.0 with $Ba(OH)_2$. Air is passed through the solution until any cysteine present has been converted to cystine (negative nitroprusside-ammonia test). The neutral solution is then boiled for 30 minutes with an excess of nitrate-free $Cu(OH)_2$ or $CuCO_3$, cooled, filtered, and the precipitate is washed. After removal of reagents, histidine is isolated as the diflavianate.

Comment: The calculation of histidine based on the nitrogen of the "histidine fraction" may be high if appreciable quantities of cystine are present in the hydrolysate. However, it appears that this cystine would be removed by the $AgNO_3$ extraction employed in the original Kossel-Kutscher method (379). Kossel and Patten (380) were cognizant of the presence of cystine in the histidine fraction.

Aberhalden *et al.* (23, 24) and Block (88) claim that there may be a considerable loss of histidine in the $Cu(OH)_2$-$CuCO_3$ precipitate especially when small volumes and small quantities of protein are employed as in the original Block adaptation of the Kossel method (86). This claim is refuted by Tristram (619).

J. Vickery and Block's Modification (650)

Principle: To insure an excess of silver ions in the *initial* precipitation of arginine and histidine, $AgNO_3$ is used in place of Ag_2O-H_2SO_4. The ammonia is removed from the lysine fraction by making the solution alkaline with NaOH rather than with $Ba(OH)_2$. This saves considerable time and effort as large quantities of $BaSO_4$ do not have to be washed. Alcohol is added to the alkaline

solution to retard foaming and to facilitate the distillation of ammonia. The lysine phosphotungstate precipitate is centrifuged, decomposed, and the lysine is reprecipitated with phosphotungstic acid. This avoids washing the heavy lysine phosphotungstate.

K. Calvery's Small Scale Adaptation of the Kossel Method (138)

Method: 1. Hydrolysis. 5 gm. of protein are refluxed with 50 ml. of 20 per cent HCl for 36 hours., 1.5 ml. of concentrated H_2SO_4 are added and the solution is evaporated to a thin syrup. This process removes almost all of the HCl.

2. Removal of Humin. The amino acid solution is made alkaline to litmus with $Ba(OH)_2$. The precipitate is removed and washed with dilute $Ba(OH)_2$.

3. Precipitation of Arginine and Histidine. This is carried out with Ag_2O-H_2SO_4 and $Ba(OH)_2$ in strongly alkaline reaction. The precipitate is decomposed with H_2SO_4 and H_2S.

4. Separation and Isolation of Histidine. Ag_2O-H_2SO_4 and $Ba(OH)_2$ are added to pH 7.4. The histidine silver precipitate is decomposed with hot dilute HCl. The solution is brought to a convenient volume and the quantity of histidine present is estimated by the Plimmer-Phillips bromine test (519) and by the Pauly-Koessler diazo reaction (278). HCl is removed from the remainder of the solution by Ag_2O-H_2SO_4 and histidine is precipitated by the addition of $\frac{2}{3}$ volume of $HgSO_4$ in H_2SO_4, prepared according to Hopkins and Cole (307) after adjusting the H_2SO_4 concentration of the histidine fraction to 5 per cent by weight. Histidine is finally isolated as the diflavianate in two crops. M.P. $= 251$–$250°$.

5. Isolation of Arginine. Arginine is precipitated with Ag_2O-H_2SO_4-$Ba(OH)_2$ as usual and is isolated as the flavianate.

6. Separation and Isolation of Lysine. After removal of Ag^+ and Ba^{++} from the lysine fraction, the solution is freed of NH_3 by $Ba(OH)_2$ and alcohol. The barium is removed with H_2SO_4 and the excess of the latter with $BaCO_3$. The filtrate is concentrated to 75 ml. and 2.8 ml. of concentrated H_2SO_4 are added to make the final acidity equal to 7 per cent. 8 gm. of phospho-24-tungstic acid in 10 ml. of hot water are added and the precipitate is allowed to stand at room temperature over night. The lysine is isolated as the picrate as usual.

Comment: Calvery (138) points out in this paper that the acidity of the histidine solution is important in the quantitative precipitation with $HgSO_4$. If the acidity is over 5 per cent by weight, the recoveries are low. He also claims that chloride interferes with the precipitation of histidine by $HgSO_4$.

Isolated quantities of the basic amino acids as compared to the total nitrogen of their respective fractions were approximately: arginine 65–75 per cent; histidine 80–100 per cent; and lysine 60 per cent.

*L. Block's Microadaptation of the Kossel Procedure (86, 88, 101)

Principle: Only single precipitations are carried out. Histidine is brought down as the silver salt at pH 7.4 ($AgNO_3$-$Ba(OH)_2$). Arginine is precipitated, after concentration of the histidine filtrate, by silver at pH 13–14 with $Ba(OH)_2$. Ammonia is removed from the arginine filtrate by NaOH and alcohol. Lysine phosphotungstate is decomposed by Winterstein's method (686) with amyl alcohol and ether according to Van Slyke (633). Arginine is isolated as the flavianate (384), histidine as the nitranilate (88, 101) and lysine as the picrate.

Reagents: 1. 8 N H_2SO_4: 224 ml. of concentrated H_2SO_4 are diluted to 1 L.

2. $AgNO_3$: 50 gm. $AgNO_3$ are dissolved in 100 ml. of water.

3. 5 per cent H_2SO_4: add 29 ml. of concentrated H_2SO_4 to 1000 ml. H_2O.

4. Amyl Alcohol-Ether mixture: 1250 ml. of ether, 1000 ml. amyl alcohol (normal or tertiary) and 50 ml. of ethanol.

5. Nitranilic acid: a 300 ml. round-bottomed, short-necked flask, equipped with a stirrer, thermometer, and dropping funnel is half immersed in a freezing mixture or dry ice-cellusolve solution. 85 ml. of fuming HNO_3 are added and the contents of the flask are cooled to 0°. To this solution, 20 gm. of hydroquinone diacetate (diacetoxyquinone) are added in small portions. The temperature is maintained at 0° to −5° during the addition of the diacetate over a period of one hour. When all of the solid is in solution, 65 ml. of concentrated H_2SO_4 are added slowly to the reaction mixture from a dropping funnel. The temperature is kept below 5°. The time of addition is about 3 hours. Nitranilic acid starts to separate out in yellow crystals after approximately two thirds of the H_2SO_4 has been added. When the addition is complete, the solution is stirred for one hour longer to ensure a maximum yield. The solution is then added to ten times its volume of finely chopped clean ice. When the ice has just melted and the temperature is about 0°, the yellow nitranilic acid is filtered off on a #3 sintered glass funnel. The filtration must take place at 0°. The sintered glass funnel, containing the nitranilic acid, is placed in a vacuum desiccator over NaOH and H_2SO_4.

The crude acid (about 30 gm.) is dissolved in a minimum quan-

tity of ice water (approximately 300 ml.) and one third its volume of cold concentrated nitric acid is added slowly. Nitranilic acid separates out in fine needles. After standing for some time at 1°, the solid is filtered off on a glass funnel and dried *in vacuo* over NaOH for several days. Yield about 50 per cent of theory or 14 gm.

We are indebted to Dr. Arnold Schein for these directions. Nitranilic acid may be purchased from the Edwal Laboratories, 732 Federal Street, Chicago, Ill.

6. Denigès' or Hopkins' Reagent: 15 per cent $HgSO_4$ in 4 or 6 N H_2SO_4.

7. Picric Acid, purified according to Benedict (62): 6 liters of H_2O are heated to boiling and 250 gm. of Na_2CO_3 are added. When this has dissolved, 500 gm. of moist picric acid are added in small portions. Before all the picric acid has dissolved, the mixture should be removed from the flame and stirred for a few minutes until there is complete solution of the picric acid. Any dirt is removed by decantation and the solution is allowed to stand at room temperature over night. The sodium picrate is filtered and washed twice with 2 liters of 10 per cent NaCl each time. The precipitate is dried between each washing. When thoroughly dried, the vacuum is turned off and 500 ml. of 1:4 HCl are poured on the filter and the mixture is stirred with a porcelain spatula. The HCl is sucked off and the process is repeated 3 more times. The picric acid on the filter is then washed with 2 liters of cold water and air dried in the dark for some time. The picric acid is recrystallized twice from hot benzene to remove any trace of NaCl.

Method: 1. Hydrolysis. 2.500 gm. of lipid free protein are hydrolyzed with 25 ml. of 8 N H_2SO_4 under reflux for 16–24 hours. A few quartz-stones and a ml. of caprylic alcohol aid in easy boiling and in the prevention of foaming. The hydrolysate is transferred to a 250 ml. Pyrex centrifuge bottle (#1055 regular) and the excess acid is neutralized with 30 gm. of $Ba(OH)_2 \cdot 8 H_2O$. The solution should turn Congo red paper black (approximately pH 3.5).

All test papers are carefully washed in order to reduce losses. Dilute $Ba(OH)_2$ or H_2SO_4 may be employed to bring the reaction to the required pH.

2. Removal of $BaSO_4$. The precipitate is centrifuged off and the supernatant liquid is filtered through 18.5 cm. Whatman folded filter paper #12 into a liter round-bottomed flask with a ground glass joint. The apparatus shown in diagram 1 is used for all vacuum distillations.

The $BaSO_4$ precipitate is washed thoroughly three or four times

with hot water by breaking up the precipitate and shaking the stoppered centrifuge bottle strongly. A few drops of caprylic alcohol are added to prevent foaming. The clear filtrate and washings are concentrated to approximately 75 ml. and are transferred into a 250 ml. centrifuge bottle.

It should be stated that all insoluble precipitates such as $BaSO_4$,
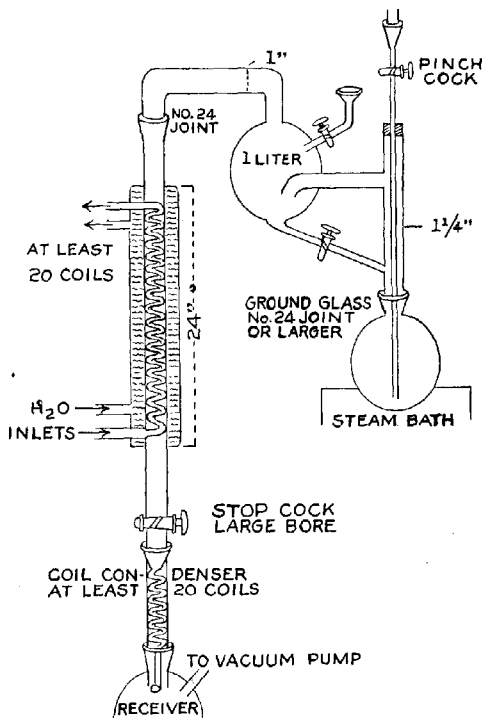
$BaCO_3$, Ag_2S, HgS, etc. should be thoroughly and repeatedly washed with boiling water. A few drops of caprylic alcohol and 1:3 H_2SO_4 can usually be added to reduce foaming and to effect better extraction.

3. Precipitation of Histidine. 50 per cent $AgNO_3$ is now added until a drop of the amino acid solution added to a small amount of dilute $Ba(OH)_2$, gives a copious precipitate of brown silver oxide (Kossel's test). Cold saturated baryta is added to the hydrolysate to pH 7.4 as indicated by a distinct blue color with bromthymol

blue. This test is also made in a porcelain spoon. The change in the p_H, as the $Ba(OH)_2$ is being added, is best followed with litmus paper. As one approaches the end point, the precipitate of histidine silver settles very rapidly. When the solution has been adjusted to p_H 7.4, it is advisable to test the reaction with phenolphthalein paper in order to be sure that the solution is definitely acid to this indicator. The precipitate is centrifuged off and the clear filtrate is *poured into the same 1 liter flask as employed for the original concentration.*

The use of the same apparatus is advisable wherever possible in order to reduce slight mechanical losses.

The histidine silver precipitate is washed once or twice with 150 ml. portions of water. The combined filtrate and washings, which contain *arginine* and *lysine*, are weakly acidified with 1:3 H_2SO_4 and concentrated *in vacuo* to approximately 50 ml. Caprylic alcohol is used in ample quantities to prevent foaming.

4. Isolation of Histidine. The histidine silver precipitate is suspended in 50 to 150 ml. of water and is acidified with 1:3 H_2SO_4 to p_H 1–2. It is sufficient to make this suspension distinctly blue to Congo paper, but care must be taken that an excess of sulfuric acid is present. The histidine silver is decomposed with H_2S. The decomposition usually takes only a few minutes and is complete shortly after the silver sulfide begins to coalesce into fairly large clumps. The H_2S is removed by aeration. The excess of H_2SO_4 is removed by the careful addition of saturated $Ba(OH)_2$ to p_H 3–4.

The reaction of the histidine solution at this point does not appear to be of great importance, but soluble barium salts must not be present.

The precipitate is removed and washed. The histidine sulfate solution is concentrated to 5 ml. The solution is filtered into a 125 ml. Erlenmeyer flask using a 9 cm. soft paper (Whatman). The still and flask are carefully rinsed with 5 to 10 ml. of water followed by two 5 ml. portions of methanol. Each washing is passed through the filter paper in succession. The histidine sulfate solution may become cloudy after the addition of methanol. The solution is cooled to room temperature and an excess of nitranilic acid is added either as a solid or dissolved in a little methanol. The precipitation of histidine nitranilate begins immediately on scratching or seeding. The solution is placed in the refrigerator over night and the precipitate is filtered on a #4 sintered glass crucible. An excess of nitranilic acid is employed. The histidine nitranilate, after washing with methanol and ether, is dried at 105° and weighed.

Histidine $= 0.403 \times$ weight of histidine nitranilate;
$$C_6H_9O_2N_3 \cdot C_6H_2O_8N_2. \quad N = 18.2 \text{ per cent.}$$

In certain instances it is desirable to purify the histidine fraction before precipitation with nitranilic acid. In these cases, the histidine sulfate solution, adjusted to pH 3 to 4, is concentrated to 100 ml. and 30 ml. of 15 per cent $HgSO_4$ in 4 N H_2SO_4 (Denigès' reagent) are added. The solution is allowed to remain in the refrigerator over night. The histidine mercury is centrifuged off and the filtrate is discarded. The precipitate is suspended in water and is decomposed with H_2S. Complete decomposition is ascertained by the deep black color of the suspension and its speedy settling when the stream of gas is stopped. The H_2S is removed by aeration and the pH is adjusted to pH 3–4. The HgS and $BaSO_4$ are centrifuged off and the histidine is isolated as the nitranilate.

Vickery (659) has suggested the use of 3,4-dichlorobenzenesulfonic acid as the precipitant for histidine. Four moles (6.8 gm. per gm. of histidine) are added to the histidine hydrochloride solution and the solution is warmed to dissolve any precipitate. It is cooled slowly at room temperature and then in the refrigerator for 24 to 40 hours. The histidine disulfonate is transferred with the aid of the *cold* mother liquor to a sintered glass crucible and washed with 3 to 5 cc. of cold 4 per cent 3,4–dichlorobenzenesulfonic acid. The crucible is dried in a desiccator over H_2SO_4 for some time, removed, and the precipitate is washed with three 10 ml. portions of ether and dried at 105°.

Histidine $= 0.2548 \times$ weight of disulfonate
Decomposition Point 273–280° $N = 6.90$ per cent (theory).

The barium salt of 3,4-dichlorobenzenesulfonic acid is insoluble.

Histidine disulfonate is soluble in 10 per cent reagent to the extent of about 95 mg. per 100 ml. It can be recrystallized from hot water.

5. Separation of Arginine. The amino acid solution, which has been brought to approximately 50 ml., is transferred to a 250 ml. centrifuge bottle and is tested for the presence of excess silver (Kossel's brown spot test). More $AgNO_3$ is added if the test is not strongly positive. The arginine silver is precipitated by the addition of hot saturated $Ba(OH)_2$. The solution is made dark red to phenolphthalein paper and then approximately 5 cc. more of baryta are added. The arginine silver precipitate is removed by centrifugation and is washed with cold saturated $Ba(OH)_2$. The filtrate and washings should be between 175 and 300 ml.

A correction based on the solubility of arginine silver of 3.6 mg. of arginine per 100 ml. may be applied (270).

The arginine filtrate contains *lysine.*

6. Isolation of Arginine. The arginine silver precipitate is suspended in 200 ml. of dilute H_2SO_4. The solution should be distinctly blue to Congo paper. The salt is decomposed with H_2S. The H_2S is removed by a stream of air and *the reaction is adjusted to black to Congo paper with $Ba(OH)_2$. The precipitate is removed by centrifuge and it is washed with hot water containing a drop of 1:3* H_2SO_4. The filtrate and washings are concentrated to approximately 15 ml. and the arginine sulfate solution is filtered through soft paper into a 125 ml. Erlenmeyer flask. The still and flask are rinsed down with small portions of water. The combined filtrate and washings, volume approximately 25–30 ml., are heated to 90° and an excess of flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid) is added either as a solid or dissolved in warm water. Within a very few minutes, the shining plates of arginine flavianate appear in the hot solution. If they do not, the solution is seeded. The flask is placed in the refrigerator over night and the precipitate is filtered on a tared sintered glass crucible #3. The arginine flavianate is washed with cold water, acetone, and ether and is dried at 110°.

$$\text{Arginine} = 0.357 \times \text{weight of arginine flavianate}$$
$$\text{M.P.} = 258–260° \text{ with darkening}$$
$$N = 17.20 \text{ per cent}; S = 6.56 \text{ per cent}$$

7. Removal of Ammonia. The strongly alkaline solution from the arginine silver precipitation is *immediately* acidified to p_H 1 with 1:3 H_2SO_4. The silver is removed by H_2S. The precipitate of Ag_2S and $BaSO_4$ is removed and thoroughly washed. The combined filtrates are concentrated *in vacuo* to 200 ml. One to 2 ml. of 0.1 per cent solution of phenolphthalein in 50 per cent alcohol are added and just enough 10 per cent NaOH so that the solution remains red after the addition of 75 ml. of ethanol. The solution is concentrated at a low temperature to approximately 10 ml. and sufficient 1:3 H_2SO_4 is added to discharge the red color. If, during the concentration, the red color disappears it may be due to insufficient alkalinity as a result of the removal of NH_3 or, as is more often the case, to destruction of the indicator. It is therefore advisable at the end of the concentration to add fresh indicator.

8. Precipitation of Lysine Phosphotungstate. Two or 4 ml. of 1:3 H_2SO_4 are now added and the solution is transferred to a 250 ml. centrifuge bottle. The still is washed three or four times with small portions of water. The final volume should not exceed 40 ml. The solution is now heated in boiling water to approximately 90° and 10 gm. of phospho-24-tungstic acid in 30 or 50 ml. of hot 2 or 5 per cent by weight of H_2SO_4 are added. The solution is placed im-

mediately in ice water and is stirred for a short time. Crystallization of lysine phosphotungstate begins almost immediately. The bottle is kept in the ice bath for 30–45 minutes. The precipitate is removed by centrifuging and it is washed carefully three or four times with 2 per cent phosphotungstic acid in 2 or 5 per cent H_2SO_4. *The volume of the lysine phosphotungstate should not exceed 150 ml.*

9. Decomposition of Lysine Phosphotungstate. The crystalline phosphotungstate is suspended in 50 to 75 ml. of water containing 4 ml. of 1:3 H_2SO_4. 50 to 75 ml. of amyl alcohol-ether mixture are now added and the suspension is stirred until all the phosphotungstate has been dissolved.

It is important to add sufficient solvent so that when the phosphotungstic acid is dissolved, the specific gravity will be less than that of the aqueous layer.

The water and solvent are transferred to a 300 ml. separatory funnel and the aqueous layer is drawn off into a second funnel of the same size. The solvent is poured into an Erlenmeyer flask and is retained for the time being. The separatory funnel should be rinsed with 10 to 15 ml. of water containing a few drops of H_2SO_4. The aqueous solution is again extracted with fresh solvent and after separation it is returned to the first funnel which has been cleaned in the meantime. The solvent is washed into the flask containing the initial solvent. The extraction of phosphotungstic acid is carried out a third time in the same manner. The lysine sulfate solution is set aside and the combined solvent solutions are poured into a separatory funnel, shaken, and the aqueous layer is drawn off. The solvent is discarded and the aqueous washing is extracted with fresh solvent.

The aqueous solutions are now combined and extracted with amyl alcohol-ether. The last solvent is extracted with a little dilute H_2SO_4.

The lysine sulfate solution, in a 250 ml. centrifuge bottle, is brought to p_H 5, i.e. red to Congo and to litmus papers. Several gm. of $BaCO_3$ are stirred in until the reaction becomes alkaline to litmus paper. The precipitate is removed and washed. The filtrate is concentrated *in vacuo* to 200 ml. in the presence of about 1 gm. of $BaCO_3$ and some caprylic alcohol. The precipitate is removed and washed and the lysine carbonate solution is concentrated to 30–50 ml. at low temperature.

10. Isolation of Lysine Picrate. The lysine solution is filtered, washed into a 100 ml. round-bottomed flask, and concentrated to a thin syrup at a *low* temperature. It is very important not to allow the lysine carbonate to become warm, i.e. over 40°. The apparatus

is rinsed down with a few drops of water and then about 10 ml. of absolute alcohol are added. The solution often becomes cloudy. An excess of purified picric acid in absolute alcohol is added to the cold lysine carbonate. Lysine picrate usually crystallizes out immediately. The flask is placed at 0° over night. The lysine picrate is filtered on a #4 sintered glass crucible and washed with cold absolute alcohol and with ether.

Lysine picrate explodes at 250° if slightly impure and 266° if pure.

$$\text{Lysine} = 0.39 \times \text{weight of lysine picrate.}$$

If the lysine picrate does not explode above 250° uncor., it should be recrystallized from a little hot water. The resulting lysine picrate should be corrected by the solubility of lysine picrate in water, 540 mg. per 100 ml. at 21–22° (Lawrow, 409) or 340 mg. per 100 ml. at 0° (Tristram, 619).

11. Isolation of Lysine Nitranilate. In the presence of large amounts of proline it is often difficult to isolate pure lysine picrate. In these cases, some success may be had with nitranilic acid. The lysine carbonate solution is concentrated to approximately 10 ml. and 25 ml. of methanol are added. An excess of nitranilic acid is introduced. The precipitate of lysine nitranilate appears in a short time. After standing in the refrigerator over night, the lysine nitranilate is filtered off on a #4 sintered glass crucible. The precipitate is washed with methanol and ether and dried at 110°. Explosion point 214° uncor.

$$\text{Lysine} = 0.387 \times \text{weight of lysine nitranilate.}$$

Comments: The method described above is the one which we have found to yield satisfactory and reproducible results with a great many proteins which differ widely in their amino acid composition and in their state of purity. However, it should be remembered that no hard and fast rules can be made for the estimation of the bases and that it is often advisable to modify the experimental conditions to meet specific needs. For example if a protein is deficient in one or more of the basic amino acids it may be necessary to employ a larger quantity of protein or to use a combination of the micro-*Kossel method with one or more of the specific colorimetric procedures to be described later.*

Our modification of the Kossel method permits two individuals to carry out two complete arginine, histidine, and lysine estimations in one and a half working days or about 12 hours. The only piece of apparatus which is not usually available in a simply equipped chemical laboratory is the rapid vacuum still shown in diagram 1. The ease with which the basic amino acids can be carried out allows

the use of control analyses with each protein to be analyzed. The control analysis is usually carried out concurrently with the unknown by adding a known quantity of arginine, histidine, and lysine to a mixture of glycine or other amino acids and starch or other carbohydrates. The mixture should be equal in nitrogen and total weight to the protein in question. 135 mg. of histidine hydrochloride hydrate, 121 mg. of arginine hydrochloride, and 150 mg. of lysine dihydrochloride are equal to 100 mg. of the free base. It has been our experience that the loss of each of the amino acids is quite constant for each protein preparation, but varies with the non-protein impurities. The loss of arginine is usually from 18 to 28 mg., of histidine from 8 to 14 mg., and of lysine from 14 to 28 mg.

Miller (451) has shown that the Block micro-modification of the large scale Kossel-Vickery method yields equally good results if the Gulewitsch correction for the solubility of arginine silver is used. It is difficult to carry out a large scale (50 gm. or more of protein) in less than two weeks.

Nitranilic acid, 2,5-dihydroxy-3,6-dinitro-p-benzo-quinone was first introduced as a precipitant for glycine by B. W. Town (617) in 1936 and for the diamino acids by Stein and Miller in 1937 (personal communication and 586). The latter investigators (586) reported that 82 per cent of the histidine present in solution was precipitated by nitranilic acid from an alcohol-water mixture such as used by Town (617) to precipitate glycine.

Mazur (442) claims that the solubility of lysine phosphotungstate is lower in 2 per cent H_2SO_4 than in 5 per cent (by weight) of this acid. The latter concentration is that generally employed.

M. Ayre's Modification (38)

Principle: The basic amino acids and ammonia are directly precipitated by phosphotungstic acid. Ammonium phosphotungstate is not decomposed by amyl-alcohol and ether and thus it can be removed by centrifugation.

Method: Four to 5 gms. of protein are hydrolyzed 36–40 hrs. with 20 per cent HCl. The hydrolysate is washed into a 250 ml. centrifuge bottle, heated, and an excess of phospho-24-tungstic acid is *added. The solution is then diluted with water until the concentra-*tion of HCl equals 5 per cent. The precipitate is allowed to form over night. It is washed twice with 2.5 per cent phosphotungstic acid in 5 per cent H_2SO_4. The precipitate is decomposed with amyl alcohol and ether (*cf.* L above). The insoluble ammonium phosphotungstate is removed and washed. The bases are separated using Ag_2O-H_2SO_4-$Ba(OH)_2$. As a rule a second phosphotungstic acid precipitation is not carried out.

*N. Tristram's Modification (619)

Principle: This is essentially the Block procedure except that the ammonia is removed as the phosphotungstate (Ayre's method) rather than by distillation.

Method: Differences in procedure from the Kossel-Block method will be given only. 1. All precipitates are washed by grinding in a mill three times unless otherwise stated. 2. $BaSO_4$ is boiled with acidulated water for 10 minutes. 3. Histidine silver is washed twice with water. 4. Arginine silver is washed twice with dilute baryta. 5. Lysine phosphotungstate is allowed to stand in a cool place (15 to 20°C.) over night.

O. The Procedure of Mourot and Hoffer (466)

Principle: Arginine and histidine are reprecipitated two or three times by silver acetate and baryta until *only* arginine and histidine are present in the precipitate. Histidine is then separated from arginine at neutrality by dilute baryta and silver acetate.

Reagents: Silver acetate (137): 900 ml. of water and 90 ml. of 20 per cent sodium acetate are heated to boiling and 150 ml. of 20 per cent silver nitrate are added. The solution is allowed to cool in a dark place. The silver acetate is filtered off, washed, and dried. It is recrystallized from water by adding two volumes of alcohol. A saturated solution of silver acetate in dilute acetic acid is used.

Method. 1. Precipitation of Arginine and Histidine. Arginine and histidine are precipitated from a small volume by Ag acetate and baryta to saturation. The precipitate is dissolved in 10 per cent acetic acid and the bases are again precipitated with silver and baryta. This is repeated two or three times.

2. Separation of Histidine. Enough dilute $Ba(OH)_2$ is added to the arginine and histidine mixture to exactly neutralize the solution and the acidity of the silver acetate to be added in excess (Kossel brown spot test). The precipitate of histidine silver, which contains some arginine, is washed three times with dilute baryta. The filtrate contains *arginine*. The precipitate is dissolved in 10 per cent acetic acid and the silver is removed with N/10 KCl. Baryta is added to neutralize the acid plus a 10 ml. excess. Then 10 ml. of silver acetate, equivalent to the 10 ml. excess of $Ba(OH)_2$ are added and the histidine silver precipitate is washed as before. Three such precipitations result in the complete separation of arginine and histidine.

Comments: No experimental results other than those on mixtures of arginine and histidine were reported. Therefore, it is not possible to evaluate this method as applied to protein hydrolysates.

DISCUSSION

Because the analytical results obtained by the Kossel method are based on the isolation of pure derivatives of the amino acids, the values must be minimal. This fact has been recognized by every investigator since it was first stated by Kossel and Kutscher in 1900. However, various means have been utilized to improve the validity of the analytical data.

Hydrolytic Losses: Possibly the principal unsolved difficulty in all amino acid analyses is, as mentioned in the introduction, destruction during hydrolysis. Kossel and Kutscher (379) and later workers have attempted to minimize these by hydrolyzing under different conditions.

Tristram (619) has observed that the yield of arginine is decreased as the quantity of carbohydrate in the preparation hydrolyzed is increased. He has proposed the following empirical correction to be applied in these cases.

Losses of Arginine in the presence of Carbohydrate

Nitrogen in per cent	17.0	16.0	15.0	12.5–13.0.
Correction factor	1.06	1.10	1.15	1.20

Arginine Losses: Gulewitsch (270), working in Kossel's laboratory, estimated the solubility of arginine silver by shaking the freshly precipitated salt for 4 hours with cold saturated $Ba(OH)_2$ in 3 per cent $Ba(NO_3)_2$. These experiments indicated that 0.036 gm. of arginine were dissolved per liter. This correction was generally used by Kossel, Osborne, and others until 1927 when Vickery and *Leavenworth (642) claimed that Gulewitsch's correction is not necessary if arginine is precipitated near pH 11. Other experience* (385, 86, 104, 451, 619, etc.) indicates the need for saturating the silver solution with baryta as advised by Kossel and the value of the Gulewitsch correction. The absolute quantity of this correction cannot be fixed with any accuracy since the solubility will vary with the nature and quantity of the other amino acids present (Miller, 451).

Losses due to the solubility of arginine flavianate in the presence of excess flavianic acid are almost negligible.

Histidine Losses: With one exception (645), it is generally agreed (23, 88, 101, 195, 381, 385) that histidine is quantitatively precipitated as the silver salt. The recent introduction of nitranilic acid and the omission of the copper purification step permits the quantitative isolation of histidine from small quantities of protein hydrolysates (Block, 101; Devine, 195).

Lysine Losses: It is remarkable that in over forty years the esti-

mation of lysine has not been changed, except for very minor details since it was first proposed by Kossel and Kutscher (379). If the crude lysine picrate does not explode above 250° uncorrected, it is advisable (643) to recrystallize the picrate from water. The correction for the solubility of lysine picrate in water at 21–22°C is 5.4 mg. per ml. according to Lawrow (409) and at 0°C is 3.4 mg. per ml. according to Tristram (619). Theorell (605) points out that the solubility of lysine picrate may amount to as much as 16 per cent of the total lysine when small quantities of protein are analyzed.

A second and more indeterminable loss is that due to the solubility of lysine phosphotungstate. Van Slyke (630) estimated that lysine phosphotungstate was soluble in excess phosphotungstic acid in the presence of approximately N HCl to the extent of 1.3 mg. per 100 ml., while Thimann (606) reported that 98 per cent of the pure base was precipitated from 10 ml. of 5 per cent H_2SO_4 by the addition of 3 ml. of 20 per cent phospho-24-tungstic acid when left at 0° over night. However, he points out that the actual correction to be employed in any experiment will be *considerably higher* and will vary with the nitrogen content of the solution. Recently Van Slyke, Hiller, and MacFadyen (639) have reported that a simple solubility correction cannot be applied, but that the solubility of the diamino phosphotungstates is a function of the quantity of the amino acid in question as well as the quantities of any other diamino acids which may be present.

Dakin (191) reported that if 300 mg. portions of lysine in solution, are treated with 15 per cent of $AgNO_3$ and N or 2 N NaOH are added *alternately* until a definite separation of brown silver oxide has occurred, 5 to 10 per cent of the base is found in the washed precipitate. It is unfortunate that this study was not extended to include the more usual $AgNO_3$-$Ba(OH)_2$ precipitation according to Kossel.

"Overall" Losses: In an effort to estimate the total loss, mechanical as well as solubility, Tristram (619) using mixtures of pure amino acids has advised the following corrections for the Kossel-Block procedure:

Arginine   3.6 mg. per 100 ml. silver baryta solution +8–20 mg.
Histidine 1–8 mg. "overall" loss   +1 mg.
Lysine   7–15 mg. "overall" loss

Or a total correction for arginine of 19 to 31 mg., for histidine 2 to 8 mg., and for lysine 7 to 15 mg. An added correction to account for the hydrolytic losses undergone by arginine and histidine has been given above.

Bolling and Block have advised the carrying out of a control analysis concurrently with each basic amino acid determination. The control consists of a mixture of the diamino acids, glycine, etc. and carbohydrate in approximately the same relative proportions of the bases to total nitrogen as is present in the protein under investigation. This permits the approximation of the "overall loss" as applied to a particular protein preparation. Some results which we have obtained by this procedure are summarized:

AMINO ACID—CARBOHYDRATE MIXTURE

Nitrogen	Arginine Loss	Histidine Loss	Lysine Loss
per cent	mg.	mg.	mg.
7.0	27.9	6.4	20.2
7.9	23.6	14.9	21.8
8.0	22.1	15.7	32.8
11.0	—	—	20.6
13.8	14.7	6.5	16.0
15.5	21.1	9.1	12.9
15.5	17.7	8.0	10.4
16.0	6.6	15.1	15.3

These results indicate that in the presence of large quantities of carbohydrates, the recovery of arginine and lysine is significantly less than in their absence. Thus we have used for protein preparations which contain 12 per cent or more of nitrogen a correction for an "overall loss" of 18 mg. of arginine and 14 mg. of lysine. If they contain less than 12 per cent N, a correction of 28 mg. for arginine and 28 mg. for lysine was used. In agreement with Tristram (620), the loss of histidine does not appear to be so closely related to the quantity of carbohydrate present during the hydrolysis, but to other factors. The average histidine loss is in the order of 9 to 14 mg.

Knight (371) reported that the addition of small amounts of histidine to tobacco mosaic virus protein before hydrolysis resulted in a recovery of 36 and 64 per cent respectively. The destructive action of carbohydrates on histidine during hydrolysis is also illustrated by the finding of 0.4 per cent of histidine in Holmes' ribgrass virus when the intact material was hydrolyzed and 0.6 per cent when the nucleic acid fraction was removed prior to hydrolysis. This confirms the earlier report of Block (cf. 101) on yeast proteins.

The tacit assumption is made that the behavior of the free amino acid is the same as that of its analogue in the protein molecule. This is not always the case, but it is believed that such recovery experiments are preferable to none at all.

2. The Separation of the Basic Amino Acids as a Group

A. *Precipitation with Phosphotungstic Acid (Hausmann,* 284)

Principle: The protein was hydrolyzed with concentrated HCl and the amide N (as NH$_3$) was determined by distillation with magnesia. The residual hydrolysate was acidified with HCl and the bases were precipitated by an excess of phosphotungstic acid in dilute HCl. After 24 hours, the precipitate was filtered off, washed with dilute reagent and the total diamino nitrogen was estimated by the Kjeldahl method.

Comment: Kossel and Kutscher (379) used 20 per cent phosphotungstic acid in 5 per cent (by weight) of H$_2$SO$_4$ while Mazur and Clarke (442) recommend 2 per cent of H$_2$SO$_4$. Winterstein (686) found that cystine was precipitated under these conditions.

Cautious treatment of the hydrolysate with activated carbon (Darco S-51) results in much purer phosphotungstates with little or no loss of base.

Although small quantities of amino acids other than the bases and cystine are now known to be precipitated by phosphotungstic acid, this procedure is often useful as a preliminary step in the determination of the diamino acids by more refined methods.

Thimann (606) found that 92 per cent of arginine, 92 per cent of histidine, 98 per cent of lysine, and 11 per cent of proline were precipitated from 10 ml. of 5 per cent H$_2$SO$_4$ at 0°. He states however, that the actual correction to be employed in any protein analysis will be considerably higher and will vary with the nitrogen content of the solution. Theorell (605) working with small quantities of protein has found that the solubilities of arginine and histidine phosphotungstates account for 11 and 13 per cent of these diamino acids present in the protein.

Van Slyke, Hiller, and Dillon (640) found that some excess of phosphotungstic acid is needed to depress the solubility of the diamino acid phosphotungstates to a minimum but variations between 25 and 100 gm. per liter make little difference. They recommend the use of 2.5 gm. of phosphotungstic acid per gm. of protein hydrolyzed and an *excess* of 50 gm. of phosphotungstic acid per liter of solution. As stated before, the strength of the mineral acid, HCl, should be 0.25 N.

Van Slyke *et al.* (640) found that while the maximum precipitation, at room temperature, of arginine and lysine was reached in a few hours, histidine and cystine required 48 hours.

Contaminating impurities are removed by dissolving the phosphotungstate precipitate in the least quantity of N NaOH and re-

precipitating by adding sufficient HCl to neutralize the alkali and enough more to bring its concentration to 0.25 N. Then, sufficient phosphotungstic acid to equal 50 gm. per liter is added and the precipitate is allowed to form at room temperature for 48 hours.

Solubility corrections for the diamino acid phosphotungstates can be calculated from figures 1 and 2 taken from the paper of Van Slyke, Hiller, and Dillon (640) in the *Journal of Biological Chemistry*.
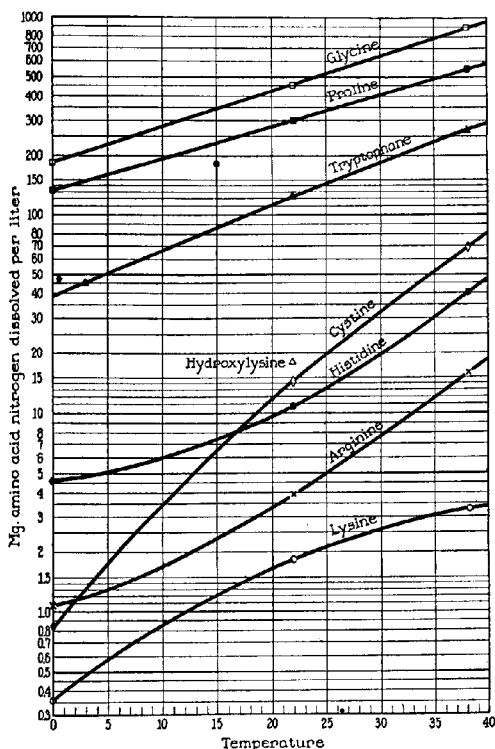
FIGURE 1

FIG. 1. Effect of temperature on the solubilities of amino acid phosphotungstates in the presence of 0.25 N HCl. The curve for histidine is not valid when the molar sum of arginine and lysine precipitated exceeds the histidine. (From: Donald D. Van Slyke, Alma Hiller, and Robert T. Dillon: The *Journal of Biological Chemistry*, Vol. 146, No. 1, November 1942.)
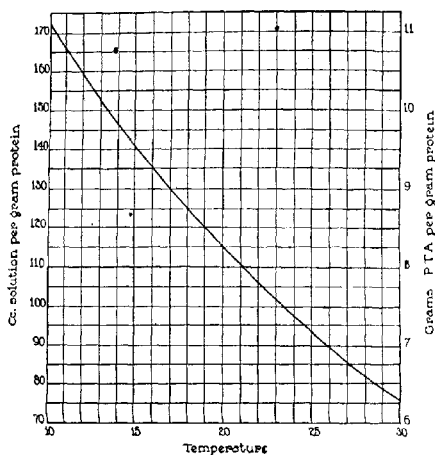
F1G. 2. Volumes of 0.25 N HCl and amounts of PTA, per gm. of protein, which appear to give optimal conditions for separation of the diamino from the monoamino acids in protein hydrolysates. (From Donald D. Van Slyke, Alma Hiller and Robert T. Dillon: The *Journal of Biological Chemistry*, 146, 1, November 1942.)

B. Phosphotungstic Acid Precipitation

(*Van Slyke*, 630, 633, 634, 639)

Principle: (a) Ammonia is removed from the protein hydrolysate. (b) The diamino acids are precipitated by phospho-24-tungstic acid in dilute HCl. (c) The phosphotungstic acid is removed by amyl alcohol and ether according to Winterstein (686) and Jacobs. (d) A portion of the amino acid solution is boiled with NaOH, only arginine and cystine liberate NH_3. (e) Sulfur is determined in an aliquot of the solution. Cystine is calculated from the sulfur content. (f) Total nitrogen, α-amino nitrogen, and ϵ-amino nitrogen estimations then permit the calculation of histidine and lysine.

Estimation of Amino Groups (628): Acetic acid reacts with $NaNO_2$ to yield $3HONO \rightleftharpoons HNO_3 + 2NO + H_2O$. The nitrous oxide is used to wash the residual air out of the apparatus. The excess NO is taken up in alkaline $KMnO_4$. CO_2 is taken up by KOH. The amino acid is then added and the following reaction takes place

$$RNH_2 + HONO \rightarrow ROH + H_2O + N_2$$
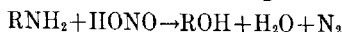
The volume of nitrogen is measured under known conditions in a gas burette.

α-Amino groups are decomposed in 5 minutes at room temperature, the ε-amino group of lysine requires 20 minutes shaking for complete deamination.

Apparatus: Diagrams of the original and improved Van Slyke apparatus have appeared in the *Journal of Biological Chemistry*, in texts on physiological chemistry and even in a number of commercial catalogues and will not be reproduced here. Folley (237) described an improved design in 1930.

Reagents: Phospho-12-tungstic Acid. The commercial acid is purified by Winterstein's method (686). The phosphotungstic acid is dissolved in water and extracted with ether. The heavy ether layer, which settles below the water phase, is then washed several times with water. The ether is removed by evaporation on the steam bath (*cf.* 640).

Amyl Alcohol. Van Slyke, Hiller, and Dillon (640) suggest the purification of commercial amyl alcohol by shaking the same with N HCl to remove the nitrogenous bases and then distilling the alcohol under reduced pressure.

Method: 1. Hydrolysis. Reflux 2 to 3 gm. of protein with 10 to 20 parts of 20 per cent HCl until the ratio of amino N to total N is maximal. It is advised to use a tared flask to permit the calculation of any loss of vapors. The excess HCl is removed by concentration *in vacuo*.

2. Amide N. A slight excess of $Ca(OH)_2$ suspension is added and the NH_3 is removed by distillation *in vacuo* at 30°. 100 ml. of alcohol are used to reduce foaming and to facilitate the removal of ammonia. The NH_3 is caught in N/10 HCl and is determined by back titration.

3. Humin N. The precipitate of $Ca(OH)_2$ is removed and washed. A nitrogen determination gives humin N.

4. Precipitation of Bases. The amino acid solution is neutralized with HCl. Then 18 ml. of concentrated HCl followed by 15 gm. of phospho-24-tungstic acid in a little water are added. The solution is diluted to 200 ml. and heated on the steam bath to dissolve the precipitate. The flask is allowed to stand at room temperature for 48 hrs. rather than 0° to decrease the precipitation of the mono-amino acids by phosphotungstic acid. The long period is required to complete the precipitation of histidine phosphotungstate. The precipitate is removed and is thoroughly washed with 2.5 per cent phosphotungstic acid in 3.5 per cent HCl.

5. Decomposition of Phosphotungstates. The phosphotungstic acid precipitate is decomposed either by dissolving in a minimal quantity of NaOH and precipitating the phosphotungstic acid with

BaCl$_2$ (630) or by extracting it with an amyl alcohol and ether mixture (633) from a suspension of the diamino acid phosphotungstates in dilute HCl. The basic amino acid solution is then brought to a convenient volume.

6. Estimation of Arginine. 25 ml. of the solution are refluxed for 6 hours with 12.5 gm. of KOH. At the end of this time the NH$_3$ is distilled into standard HCl. The solution is diluted with 100 ml. of water. NH$_3$−N.=$\frac{1}{3}$ of arginine N.

7. Estimation of Cystine. As cystine gives 17–18 per cent of its N as NH$_3$ during the arginine estimation it is important to know the quantity of this amino acid. This is determined by a Benedict-Denis sulfur method.

8. Amino and Total Nitrogen. Estimations of α- and ϵ-amino N carried out in the Van Slyke apparatus permit the calculation of histidine and lysine.

Histidine = 1.5 Total Non Amino N − 1.125 Arginine N

Lysine   = T.N. − (Arginine N + Cystine N + Histidine N)

or   Lysine   = 2 × ϵ-Amino N

Comments: Van Slyke (630) determined the solubility of arginine, histidine, and lysine phosphotungstates under the conditions of this method to be per 100 ml.:

Arginine N = 1.6 mg. or Arginine = 5 mg.

Histidine N = 1.9 mg. or Histidine = 7 mg.

Lysine   N = 0.25 mg. or Lysine = 1.3 mg.

In 1941 Van Slyke, Hillér, and MacFadyen (639) said that the diamino acids are precipitated as mixed salts and not as individual substances each with its own individual solubility. The effect on a given diamino acid present in small amount or forming a more soluble phosphotungstate than the average of the group, is to diminish the loss of such an amino acid below the loss that would be calculated from the solubility of its isolated phosphotungstate. It is also suggested that the bases be precipitated at approximately 0.25 N HCl rather than in approximately N HCl as suggested in 1911 (*cf.* A above).

Van Slyke (628) also showed that cystine gave 107 per cent of the expected quantity of nitrogen. This correction was therefore used until 1937 when Kendrick and Hanke (359) found that the use of 2 per cent KI in the acetic acid reduced the over production of N by cystine and by glycine to the expected quantities.

Plimmer and Rosedale (518, 520), in a careful discussion of the Van Slyke method, point out (1) histidine can also yield more or less NH$_3$ on heating with strong alkali, (2) it is immaterial whether

the phosphotungstates are precipitated at room temperature or 0°, (3) slight differences in NH_2-N values make great differences in the final results, (4) the solubility factors, which have little meaning in complex mixtures anyway, may be neglected as the method is chiefly comparative, and (5) "the common practice of returning the data to two decimals is entirely misleading."

Improvements in the estimation of arginine have been made by Koehler (374) and in the general procedure by Narayana and Sreenivasaya (471) and by Cavett (151). It is obvious that any of the amino acids precipitated by phosphotungstic acid can be estimated by one or more of the specific gravimetric, colorimetric, or gasometric methods. It is often unnecessary to remove the phosphotungstic acid, simple solution may suffice for further determinations.

Van Slyke in 1915 (633) stated that the method was designed for use only with proteins not accompanied by other classes of substances, particularly nitrogenous substances which would obviously falsify the interpretation of the results. Unfortunately this advice has not always been heeded and there are many estimations of arginine, histidine, and lysine in the literature, which as a consequence, have little value.

C. Separation of the Diamino Acids Electrolytically
(27, 172, 182, 261, 393)

Historical: Although the employment of electrodialysis for the separation of the diamino acids from the other components of a protein hydrolysate was disclosed as early as 1912 in a Japanese patent, the use of this procedure as a preliminary step in the quantitative estimation of arginine, histidine, and lysine appears to have originated with Kuhn and Desnuelle (393) in 1937.

Apparatus: A three celled electrodialysis apparatus of 100 ml. capacity or larger is used with platinum or carbon anodes and Pt. cathodes. The contents of the middle chamber should be mechanically stirred. All three chambers are cooled by a stream of water passing through glass coils. Membranes are of cuprophan (393) or linen coated with 4 per cent HCHO hardened gelatin (anode) and parchment paper (cathode) (27), etc. The current used can be from 110 to 220 v., 0-1 amp. The current is stabilized by passing through a 100 or 250 w. lamp which is connected in series with the electrolytes. The levels of the fluid in each cell must be the same.

Method: 1. Removal of Mineral Acid (27). The protein is hydrolyzed with 1:1 HCl, diluted to 100 ml. and placed in the center compartment. A 7-8 hour electrolysis places the amino acids in the cathode chamber. The complete removal of the bases from the

center cell is ascertained by testing with Sakaguchi or phospho-
tungstic acid reagents and is indicated by a sudden drop in current
(Albanese, 27).

2. Separation of the Diamino Acids. The catholyte of the first
electrolysis is brought to pH 5.6–5.8 (brom cresol purple and methyl
red) (27) or pH 6 (brom thymol blue) (261) placed in the middle
compartment and again electrolyzed until the middle cell gives a
negative Sakaguchi test (393).

3. Determination of Arginine, Histidine, and Lysine. This is
carried out by the Kossel, Van Slyke, or a combination of the
specific tests for each of the diamino acids.

Comments: Preliminary electrodialysis serves the useful purpose
of separating the diamino acids from carbohydrate and nonbasic
amino acid impurities, and in contrast to phosphotungstic acid
precipitation, electrodialysis may be made quantitative. Electro-
dialysis is especially useful in those hydrolysates which contain
large quantities of proline which makes the isolation of lysine pic-
rate very difficult and those which contain large amounts of
carbohydrate breakdown products which may interfere with the
isolation of arginine and in the precipitation of the diamino acid
phosphotungstates.

Gordon, Martin, and Synge (261) advise that the electrodialysis
be repeated three times in order to separate completely the bases
from the monoamino acids. These investigators as well as Kuhn and
Desnuelle (393), Albanese (27) Csonka (182), and others have suc-
cessfully used this procedure.

D. Separation of the Diamino Acids by Selective Adsorption
(676, 107, 622)

Historical: In 1923, J. C. Whitehorn published the interesting
observation that the synthetic zeolite, "Permutit," $2SiO_2$, Al_2O_3,
$Na_2O \cdot 6H_2O$, which Folin had used to remove ammonia from neu-
tral aqueous solutions, would take up arginine, histidine, and lysine.
The adsorbed bases could then be exchanged by treating the
"Permutit" with saturated KCl. The following equation was pro-
posed:

$$(NH_4Cl) \times (Na\ permutit) = K(NaCl) \times (NH_4\ permutit)$$
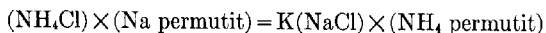
Although "Permutit" exchange should be a satisfactory method
for removing the diamino acids and ammonia from the other prod-
ucts of protein hydrolysis, it has not been widely used as a prelimi-
nary step in amino acid analysis because of the need of a strong
sodium or potassium chloride solution to remove the bases from the
zeolite. To the authors' knowledge, only in the case of Dubnoff's

(199) modification of the Sakaguchi test (562) has base exchange on "Permutit" been satisfactorily used as a preliminary step in the determination of one of the diamino acids. This reagent may, however, prove more useful in the future.

Activated earths such as fuller's earth and Lloyd's reagent have been widely used in the concentration of the basic vitamins of the B group since their introduction by Seidell. However, it was not until the recent paper by Turba (622) that these substances were proposed as a reagent for the quantitative separation and isolation of the diamino acids.

3. TURBA'S METHOD FOR THE ISOLATION AND SEPARATION OF THE BASIC AMINO ACIDS (622)

Reagents: Bleaching Earths: Filtrol-neutrol and Floridin XXF extra.

Pyridine-Sulfuric Acid: 500 ml. of N H_2SO_4 are mixed with 100 ml. of pyridine and 400 ml. of water are added.

Method: It is necessary to work rapidly and therefore a thin layer of the adsorbent should be used.

1. Separation of Histidine from the Monoamino Acids. 2 ml. of a solution containing 5 to 10 mg. of histidine and the same quantity of a monoamino acid, are added slowly to a thin layer of 3 gm. of Filtrol-neutrol. The quantitative transfer is made with a few ml. of water. The adsorbed material is then thoroughly washed with 30 to 60 ml. of H_2O to remove the monoamino acids. The histidine can then be eluted with 80 ml. of pyridine-sulfuric acid mixture or with dilute $Ba(OH)_2$.

2. Separation of Arginine and Histidine. A mixture of 20 mg. each of arginine and histidine are adsorbed on 12 gm. of Floridin XXF. The histidine is then washed out with 200 ml. of water during the course of 1 hour. The arginine is then eluted with pyridine-sulfuric acid mixture or $Ba(OH)_2$.

3. Separation of Arginine and Lysine. An aqueous solution containing 25 mg. of arginine and the same quantity of lysine is added to 25 gm. of filtrol-neutrol. The lysine is extracted with 200 ml. of m/6 KH_2PO_4 and the arginine is subsequently eluted with 100 ml. of C_6H_5N-H_2SO_4 mixture.

Comment: The possibilities for the use of specific adsorbents, synthetic zeolites, and especially synthetic base exchange resins for the separation and preparation of amino acids are only just being recognized. The recent publication of Block (107) has shown that the synthetic ion exchange resins may be used to remove completely the diamino acids from solution.

PART II

DIRECT DETERMINATION OF ARGININE

1. Oxidation to Guanidine (Orglmeister, 487)

Principle: The neutralized protein hydrolysate is oxidized with $Ca(MnO_4)_2$. After removal of the precipitate, guanidine is isolated from the neutral solution by precipitation with sodium picrate.

Comment: The results obtained by Orglmeister in 1905 are not much lower than those by more recent methods. This procedure for arginine, although admitted by Orglmeister not to be as accurate as the Kossel methods, was much easier and faster to carry out. Only 4 gm. of protein were needed instead of 50 to 100 gm. by the Kossel procedure.

As far as we know this is the earliest method for the quantitative estimation of an amino acid by an oxidative procedure although Schulze, Barbieri, and Bosshard (574) oxidized phenylalanine to phenylacetaldehyde for a qualitative confirmation of the presence of the former.

2. The Diacetyl Reaction

A. Harden-Norris Test (282)

Principle: In 1911, Harden and Norris showed that arginine and certain other guanido compounds gave a red color with a green fluorescence when treated in dilute alkali with diacetyl, $CH_3COCOCH_3$.

B. Lang's Modification of the Harden-Norris Reaction (399)

Principle: Acetylbenzoyl is used in place of diacetyl. Hydroxylamine is employed to remove the excess ketone.

Method: 1. Hydrolysis. Hydrolyze 200 mg. of protein with 4 ml. of 20 per cent HCl for 20 hours. Dilute the solution to 5 ml. and remove the humin by shaking with kaolin. Use 1 ml. of the clear filtrate, which should contain from 0.2 to 0.8 mg. of arginine, per determination.

2. Development of Color. To 1 ml. of hydrolysate add 1 ml. of 60 per cent KOH and 0.2 ml. of 1 per cent alcoholic solution of freshly prepared acetylbenzoyl. Warm for 15 minutes in boiling water. Cool and add 1 ml. of 5 per cent $NH_2OH \cdot HCl$ and dilute to 10 ml. Read after 30 minutes with filter 530 mu. Extraneous colors are compensated for by using a "blank" of all reagents except acetylbenzoyl.

Comment: This method has been adversely criticized by Jean (317).

3. HYDROLYSIS OF ARGININE TO ORNITHINE AND UREA

A. *Jansen's Procedure* (316)

Principle: In 1916, Jansen proposed the determination of arginine in a protein hydrolysate by a method which was based upon the production of urea by the action of arginase on arginine and then by the formation of ammonia from urea by urease.

B. *Bonot and Cahn's Modification of Jansen's Method* (114)

Principle: Arginine is decomposed into urea and ornithine by arginase. The urea is determined quantitatively by xanthydrol.

Reagents: Arginase. A dog is killed by exsanguination and the liver is perfused with Ringer's solution. The liver is ground with sand and the juice is expressed with an hydraulic press. The proteins are precipitated and washed with acetone. The precipitate is dried at 37°, ground, and preserved over H_2SO_4. The activity of the liver powder must be checked against arginine carbonate.

Method: 1. Hydrolysis. 1 to 3 gm. of protein are hydrolyzed for 48 hrs. with 20 per cent HCl. The hydrolysate is then autoclaved with 35 per cent HCl for $1\frac{1}{2}$ hrs. The excess HCl is removed and the amino acids are decolorized with charcoal. The solution is adjusted to *p*H 9.9 with NaOH.

2. Hydrolysis of Arginine. An excess of liver powder is added to an aliquot of the protein hydrolysate and incubated at 37° for 72 hours. Toluene is used as the preservative. The digest is weakly acidified with acetic acid and is evaporated to dryness at 60°. The residue is taken up in 70 per cent acetic acid and any precipitate is removed and washed.

3. Precipitation of Urea. Twice the calculated quantity of 10 per cent xanthydrol in methanol is added and the solution is allowed to stand 10 hours or longer. Dixanthydrylurea (Fosse, 241) is filtered off and washed with 50 ml. of methanol previously saturated with dixanthydrylurea and dried at 105°C.

$$\text{Arginine} = 0.414 \times \text{weight of dixanthydrylurea}$$

C. Graff's Modification of the Jansen-Bonot Procedure (263)

Reagents: Preparation of Xanthydrol. 25 gm. of xanthone are suspended in 200 ml. of ethanol. The ketone is reduced with mercury amalgam (0.9 gm. Na in 980 gm. of Hg) at 50 to 60°. It is shaken until the xanthone has dissolved and then 10 minutes longer. The solution is centrifuged and filtered through dry paper

into 2 liters of cold water. The precipitate is removed and washed with ice water until free of NaOH and dried in air. The xanthydrol is recrystallized from 100 ml. of absolute alcohol at $-10°$. Yield 75 per cent or 19 gm.

Tanret's $HgCl_2 \cdot KI$ Reagent. To 1.35 gm. of $HgCl_2$ in 25 ml. of H_2O, 3.32 gm. of KI in 25 ml. of H_2O are added and the solution is diluted to 60 ml. Then 20 ml. of glacial acetic acid are added.

Method: 1. Hydrolysis with Arginase. 5 to 10 ml. of a neutralized protein hydrolysate, pH 6.6, are treated with 5 to 10 drops of arginase solution at 37° over night. The solution is acidified with 3 drops of acetic acid and the proteins are precipitated with 5 to 10 drops of Tanret's reagent. The suspension is diluted to 12.5 ml. and centrifuged.

2. Precipitation of Urea. A 5 or 10 ml. aliquot of this solution is stirred with an equal volume of acetic acid containing 10 drops of 10 per cent xanthydrol in methanol. After 5 minutes, 1 per cent xanthydrol in acetic acid equal in volume to that taken for the determination is added while stirring for 30 minutes, the precipitate is filtered and washed with methanol and acetic acid saturated with dixanthydrylurea.

Comment: The aliquot used should not contain more than 1.5 mg. of urea. Kiech, Luck, and Smith (360) determined the dixanthydrylurea by $K_2Cr_2O_7$ titration.

D. *Hunter and Dauphinée's Modification of the Jansen Method* (313)

Principle: Arginine is hydrolyzed to ornithine and urea by arginase and the urea is hydrolyzed to ammonia by urease. The NH_3 is determined by titration.

Reagents: Arginase. Mix a weighed quantity of freshly ground ox or calf liver with 75 per cent glycerol equal in ml. to the weight of the tissue in gm. Shake 10 minutes. Place in a 62–65° water bath. Stir until the contents are at 58° and hold at this temperature for 5 minutes. Cool the suspension under the tap and filter on soft folded paper. After 12 hours, adjust the filtrate to pH 7 with NaOH. Determine the activity of the arginase solution. If the preparation contains deaminase, it is advisable to discard it rather than correcting for the same.

One Arginase Unit is that quantity of liver solution which will liberate 0.5 mg. of urea N from 10 mg. of arginine N in 30 minutes at 37° and pH 8.4. A good preparation will contain 80 to 100 units per ml. Keep cold. Use 1 ml.

Method: 1. "Blanks." It is necessary to run blank controls. Corrections must be applied for (a) ammonia and amide N of the protein, (b) ammonia of the liver extract, (c) NH_3 from urease, (d)

NH_3 from urea of liver extract, (e) NH_3 from deaminase in liver extract if such a preparation is used.

2. Hydrolysis. Hunter and Dauphinée (313) found that the entire arginine content of gelatin becomes susceptible to arginase action after 3 hours hydrolysis with 20 per cent HCl and that longer boiling distinctly diminished the yield of arginine. There is an increase in ammonia formed in the hydrolysate the longer it is boiled. Therefore, it is advisable to hydrolyze several samples for varying lengths of time, 5 to 24 hours, after which the HCl is removed by concentration *in vacuo* and the amino acid solution is neutralized to *p*H 6.7 with NaOH. The hydrolysate is diluted so that 5 cc. of solution contains 20 to 40 mg. of arginine.

3. Action of Arginase. Four 5 ml. portions of the hydrolysate are treated in Van Slyke-Cullen urea tubes with 2 ml. of 0.25 M Na_2PO_4+phenolphthalein+N NaOH to light pink (*p*H 8.4). To each tube 1 ml. of arginase solution (liver extract) and a little toluene is added. The tubes are incubated at 37° for 12 to 24 hours. A drop of phenol red is added and the *p*H is adjusted to 6.8.

4. Action of Urease. One ml. of urease is added to two of the tubes and after standing for 1 hour, 9 ml. of saturated K_2CO_3 are introduced, and the NH_3 is aerated as usual into standard acid. Caprylic alcohol is used to control the foaming.

5. Amide Blank. This contains the hydrolysate, but no arginase. Urease is added and the NH_3, after liberation, is aerated into standard acid.

6. Enzyme Blank. One ml. of arginase solution is added to 3 ml. of H_2O, the *p*H is adjusted to 6.8 and urease is added as before. The NH_3 is determined.

Comment: If the proteins yield much humin, this and the amide N can be removed by treating the neutralized hydrolysate with a slight excess of $Ca(OH)_2$.

Hunter and Dauphinée (313) found that the quantity of arginine in phosphotungstic acid precipitates corresponded quite closely to that obtained directly if Van Slyke's solubility correction for arginine phosphotungstate was used.

Limits of accuracy: arginase 98.5 per cent recovery, urease 99.4 per cent recovery.

E. Hunter and Pettigrew's Modification (314)

Principle: Hunter and Pettigrew have reverted to Jansen's original procedure in so far that they add arginase and urease simultaneously. Ammonia is then determined manometrically according to Peters and Van Slyke (516).

Reagents: Urease. 100 ml. of glycerol extract of jack-bean meal

plus 2.5 ml. of phosphate buffer (27.8 gm. of $Na_2HPO_4 + 27.0$ gm. of KH_2PO_4 in 100 ml. of water).

Enzyme Mixture. 10 ml. of arginase solution (*cf.* Hunter and Dauphinée above) +4 ml. of urease +75 per cent glycerol to 20 ml. This solution is allowed to age for 24 hours.

Procedure: The enzyme mixture is allowed to react on the neutralized hydrolysate, (*p*H 7), for 3 to 12 hours at room temperature. Ammonia is then determined according to Van Slyke's manometric method.

Comment: This procedure appears to be very accurate. The paper of Hunter and Pettigrew (314) should be examined for details.

4. PRECIPITATION OF ARGININE WITH FLAVIANIC ACID

A. The Method of Kossel and Gross (384, 386)

Principle: In 1924, Kossel and Gross (384) found that arginine is quantitatively precipitated from a weakly acid protein hydrolysate by the addition of a large excess of 2,4-dinitro-1-naphthol-7-sulfonic acid.

Method: 1 gm. of protein is hydrolyzed with HCl or H_2SO_4 and the reaction is adjusted to weakly acid to Congo paper (386). Arginine is precipitated by the addition of 4 equivalents of flavianic acid in concentrated aqueous solution. It is advisable to stir frequently during the first few hours. The precipitate is allowed to form in the cold for 2 or 3 days. The yellow precipitate is filtered off and washed with cold dilute flavianic acid. This precipitate is dissolved in hot water by the aid of a little dilute ammonia and is heated on the steam bath for 2 hours; total volume 100 to 150 ml. of water containing a small amount of flavianic acid. The arginine flavianate is filtered off after cooling for some time. The orange precipitate is washed with ice water, alcohol, and ether and is dried at 95 to 100° to constant weight.

Comment: Kossel and Gross (384) claim that the yields of arginine determined by the direct method were higher than by the silver precipitation procedure.

ARGININE IN PROTEINS

Protein	Direct Method per cent	Silver-Baryta Method per cent
Edestin	12.4	12.3, 12.7
Gelatin	8.2	7.2–8.3
Salmin	44.2	43.8, 44.2
Casein	4.5	4.7
Arachin	15.4	14.5

All proteins have been calculated to 16.0 per cent of nitrogen. Their results, presented above, do not however, give much support to this thesis.

Arginine flavianate is soluble to the extent of 0.0177 per cent at 19°C and 0.57–0.58 per cent in boiling water. In the presence of excess flavianic acid, it is practically insoluble.

Furth and Deutschberger (251) apparently failed to redissolve the original yellow arginine precipitate and claimed that the procedure of Kossel and Gross did not yield arginine flavianate of sufficient purity, especially from hydrolysates of tissue proteins. They, therefore, suggested a preliminary precipitation with phosphotungstic acid and attempted to correct for the solubility of arginine phosphotungstate and other losses.

* B. Vickery's Modification of the Kossel-Gross Direct Method (656)

Principle: Vickery (656) showed that the yellow precipitate of arginine with flavianic acid, which is formed in the cold, is arginine diflavianate. This is converted into arginine monoflavianate by heating with water.

Method: 1. Hydrolysis. 20–25 gm. of protein are hydrolyzed with 500 ml. of 20 per cent HCl. The excess acid is removed by concentration *in vacuo*. The syrup is diluted to a convenient volume and the protein is calculated from a nitrogen determination.

2. Decolorizing with Norite. The amino acid solution is boiled with 5 gm. of norite and the carbon is washed twice with boiling water. The filtrate is concentrated so that 50 ml. aliquots may be used for the precipitation.

3. Precipitation of Arginine Diflavianate. To a 50 ml. aliquot containing 5 gm. of protein, 4 to 5 moles of flavianic acid are added at room temperature. The precipitate is allowed to form at ice box temperature for 4 days and stirred once each day. The yellow precipitate is filtered on a crucible and washed with 30 ml. of water saturated at room temperature with arginine flavianate.

4. Precipitation of Arginine Monoflavianate. The diflavianate is stirred with a little hot water and 5 N NH_4OH is added drop by drop until the precipitate has all dissolved. The suction is applied and the crucible is washed with water. The solution (40 ml.) is heated to boiling and N H_2SO_4 is added with stirring in slight excess of the ammonia used. The solution is chilled over night and the arginine flavianate is washed with water previously saturated with the salt, and with alcohol and ether. The precipitate is dried at 105°. Arginine flavianate contains 6.56 per cent of sulfur.

Solubility of Arginine Monoflavianate in Water
at 100° 575. mg. per 100 ml. (Kossel-Gross)
at 24° 20.0 mg. per 100 ml. (Vickery)
at 19° 17.7 mg. per 100 ml. (Kossel-Gross)
at 7.5° 11.8 mg. per 100 ml. (Vickery)

Comment: In the presence of proteins which yield large quantities of histidine, the arginine flavianate may be contaminated with histidine diflavianate (659).

5. The α-Naphthol-Hypochlorite Reaction

Historical: In 1925, Sakaguchi (562) found that arginine and other unsubstituted and monosubstituted guanidino compounds gave a red color when treated in alkaline solution with NaOCl and α-naphthol. This reaction is sensitive to one part in one million of arginine.

A. Method of Sakaguchi (562, 563)

Reagents: 5 per cent NaOCl is prepared by allowing 300 to 330 ml. of HCl (sp. gr. 1.17) to drop on 50 gm. of $KMnO_4$. The resulting Cl_2 is washed with H_2O and passed into 1 liter of 10 per cent NaOH.

α-Naphthol solution is made by dissolving 100 mg. in 100 ml. of 70 per cent ethanol.

Method: Make 3 ml. of the arginine solution strongly alkaline with NaOH. Add 2 drops of α-naphthol followed by 1 drop of NaOCl. The red color forms quickly.

Comment: The test is good for qualitative work, but to obtain quantitative results it is necessary to vary the amounts of α-naphthol and NaOCl until a maximum color is achieved.

Sakaguchi formulated the reaction with guanidoacetic acid thus:

$$
\begin{array}{l}
\quad\quad\quad /OC_{10}H_6Cl \\
N \\
\quad\quad\quad \backslash OC_{10}H_7 \\
| \\
C{=}NH \\
| \\
NHCH_2COOH
\end{array}
$$
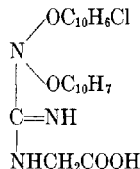
B. Weber's Modification (673)

Principle: NaOBr is substituted for NaOCl and urea is used to destroy the excess NaOBr which makes the colored arginine compound unstable.

Reagents: 0.02 per cent α-naphthol: dilute 20 ml. of 0.1 per cent α-naphthol in 95 per cent ethanol to 100 ml. with water.

NaOBr: 2 gm. of bromine in 100 ml. of 5 per cent NaOH. Keep cold and dark. Prepare every two weeks.

Urea: 40 per cent.

Method: To 5 ml of unknown (0.005 to 0.05 mg. of arginine) add 1 ml. of 10 per cent NaOH and 1 ml. of α-naphthol. Cool 2–3 minutes at 4°. Add 0.1 to 1.0 ml. of cold NaOBr, shake, and add 1 ml. of urea within 4 to 6 seconds of introducing the NaOBr. Read in 5 minutes.

Comment: It is necessary to determine by trial how much NaOBr must be added to get maximum color. Increments of 0.1 ml. are advised. The quantity of NaOBr depends on the kind and amounts of substances other than arginine which are present in solution. As the amount of color developed is *not* proportional to the quantity of arginine, it is necessary to have the volumes and concentration of the unknown and the standard closely similar. Once the color has been developed, the solution may be diluted with water, alcohol or 1:4 glycerol-ethanol mixture (Dumazert and Poggi, 200).

Weber reported the test to be sensitive to 0.0004 mg. of arginine per ml. while Jean (317) points out that the method is accurate over a range of 0.015 to 0.035 mg. of arginine only.

Histidine, tyrosine, tryptophane, ammonia, metal salts, etc. inhibit the reaction.

Jorpes and Thorén (345), Dumazert and Poggi (200), Thomas, Ingalls, and Luck (607), Dubnoff (198, 199) and others have made minor modifications in the Sakaguchi-Weber procedure. These usually consist in changes in the concentration of the reagents or in the time, temperature, and order of their addition. It has been proposed (127), that the inhibition due to ammonia and other substances can be compensated for by plotting the apparent arginine content found against the quantity of protein used for analysis. If the curve is extrapolated to "zero" protein concentration, the "true" value of arginine is claimed to be obtained. This general procedure has been employed previously by Kraus-Ragins for tryptophane and by Bushill, Lampitt, and Baker in the estimation of cystine.

C. Dubnoff's Modification (199)

Principle: Arginine is freed from guanidoacetic acid and other substances by preliminary absorption on·Permutit (base exchange).

Apparatus: Absorption column, a glass funnel, the upper part of which is 15 mm. external diameter, with a 100 mm. long stem of 7 mm. diameter. The lower end of the stem is slightly constricted to hold a cotton plug. 0.9 gm. of Permutit "according to Folin" are used.

Procedure: 5 ml. of solution are passed slowly through the Permutit which is washed with 5 ml. of 0.3 per cent NaCl. The arginine is eluted with 10 ml. of 10 per cent NaCl.

A 2 ml. aliquot of this solution is used for the determination of arginine by the Sakaguchi-Weber reaction. To 2 ml., 0.5 ml. of cold α-naphthol-urea (0.2 per cent α-naphthol in absolute alcohol diluted with 4 volumes of 10 per cent urea before use) solution are added. After standing for 2 minutes, 0.2 ml. of NaOBr (0.66 ml. of Br_2 in 100 ml. of 5 per cent NaOH) are added. The solution is allowed to stand 20 minutes at 0°. It is warmed quickly to room temperature and read with a 525 mu filter.

Comment: Ammonia, 60 mg. per cent; histidine, 5 mg. per cent; tyrosine, 8 mg. per cent; tryptophane, 8 mg. per cent; creatine, 20 mg. per cent and urea, 2000 mg. per cent do not interfere. Higher amounts of histidine and tryptophane do.

D. *Macpherson's Modification* (434)

Principle: Repeated submaximal oxidation with NaOBr.

Method: Dilute a solution containing 0.04 to 0.40 mg. of arginine to 10 ml. with water. Add KOH drop by drop to alkaline to litmus. Then add 1 ml. of 10 per cent KOH and 1 ml. of 40 per cent urea. Mix and cool under the tap. Add 1 ml. of KOBr (2 gm. Br_2 in 100 ml. of 5 per cent KOH), with mixing. Stand at room temperature 2 to 3 minutes. Repeat the urea and KOBr additions as before. Dilute to 25 ml. with water. Stand at room temperature for 10 to 15 minutes. Read with a 530 mu filter.

Comment: This appears to be the simplest modification of the Sakaguchi-Weber method. However, no analytical results were given. A combination of Permutit absorption (Dubnoff) and Macpherson's method may be the best application of the Sakaguchi reaction.

The influence of hydrolysis on the quantity of an amino acid found in the hydrolysate cannot be stressed too often. Thus, Roche and Blanc-Jean (551) have confirmed the finding of Hunter and Dauphinée (313), who used a different method, that the number of arginine groups decrease with increasing time of hydrolysis. Using the Sakaguchi-Dumazert method, the former investigators (551) report that from 15 to 35 per cent of the total guanido groups are lost after 24 hour hydrolysis with HCl.

CHAPTER I

PART III

DIRECT DETERMINATION OF HISTIDINE

1. The Diazo Reaction (Pauly)

Historical: In 1904, Pauly (513) reported that of all the known amino acids which occur in protein hydrolysates only tyrosine and histidine give an intense red color when treated in alkaline solution with freshly diazotized sulfanilic or similar acids. The test is sensitive to one part in 100,000.

The following structures were proposed (514):

Tyrosine with diazotized arsanilic acid

$$\text{HOOC}\cdot\text{CH}\cdot\text{CH}_2 \underset{\text{NH}_2}{\overset{}{\mid}} \left\langle \begin{array}{c} \text{N:NC}_6\text{H}_4\text{AsO}_3\text{H}_2 \\ \text{OH} \\ \text{N:NC}_6\text{H}_4\text{AsO}_3\text{H}_2 \end{array} \right.$$
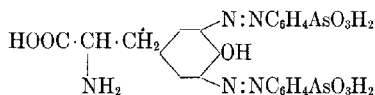
Histidine with diazotized arsanilic acid

$$\text{HOOC}\cdot\text{CH}\cdot\text{CH}_2\cdot\underset{\text{NH}_2}{\overset{\text{N}=\text{C}}{\mid}}\text{C}=\text{C} \begin{array}{c} \text{N:NC}_6\text{H}_4\text{AsO}_3\text{H}_2 \\ \text{NH} \\ \text{N:NC}_6\text{H}_4\text{AsO}_3\text{H}_2 \end{array}$$
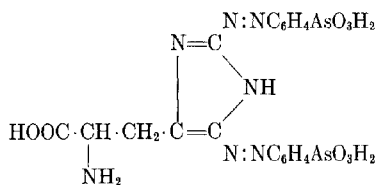
A. Weiss and Ssobolew's Modification of the Pauly Reaction, 1913 (674)

Principle: The Pauly method is used in the absence of tyrosine, i.e. negative Millon's test. The histidine may be precipitated by $HgCl_2$–Na_2CO_3 at neutrality or by silver nitrate-barium hydroxide at weakly alkaline reaction (*cf.* Kossel procedures).

Reagents: Diazotized Sulfanilic Acid. Mix 4 gm. of sulfanilic acid with 40 ml. of concentrated HCl plus 400 ml. of water. Add 2 volumes of 0.5 per cent $NaNO_3$ just before use.

Procedure: To 10 ml. of histidine solution (1:10,000) add 1.5 ml. of p-diazobenzene sulfonic-acid reagent and 3 ml. of 10 per cent Na_2CO_3. Read at optimum color development.

B. Koessler and Hanke's Adaptation of the Pauly-Weiss Test
(375, 278, 279)

Reagents: p-Diazobenzene sulfonic acid: Pipette 1.5 ml. of sulfanilic acid solution (Weiss and Ssobolew) and 1.5 ml. of 5 per cent $NaNO_2$ into a 50 ml. volumetric flask. Cool in ice 5 minutes and add 6 ml. more of $NaNO_2$. Mix well. Cool in ice 5 minutes longer. Dilute to 50 ml. Keep in ice bath. Age for 15 minutes after diluting.

Method: 1A. Hydrolysis with HCl (278). 1 gm. of protein is hydrolyzed with 20 per cent HCl. The excess acid is removed by concentration *in vacuo* and the humin and NH_3 are removed with $Ca(OH)_2$. The basic amino acids are precipitated with phosphotungstic acid according to Van Slyke (630, *cf.* above). The precipitate is filtered off and washed with 200 ml. of solution containing 18 ml. of 37 per cent HCl and 15 gm. of phospho-24-tungstic acid which has previously been saturated with histidine phosphotungstate. The washed precipitate is dissolved in dilute NaOH.

1B. Hydrolysis with H_2SO_4 (279). The protein is hydrolyzed with 8 N H_2SO_4 and the excess acid is removed with baryta. Histidine is precipitated with Ag_2SO_4 and baryta according to the Kossel methods (*cf.* above). The histidine silver precipitate is decomposed with hot dilute HCl.

2. Coupling with Diazotized Sulfanilic Acid (375). (1-X) ml. of $H_2O + 5$ ml. of 1.1 per cent Na_2CO_3 are pipetted into a reading tube or cuvette. 2 ml. of p-diazobenzene sulfanilic acid reagent are added. The time is measured to the exact second. After mixing for 20 seconds, X ml. of unknown are added exactly 60 seconds after the reagent has been introduced. The color is read at the maximum intensity, usually 6 seconds.

Comments: The method is sensitive to 0.01 mg. of histidine.

Under the conditions described above, Hanke and Koessler (278) found that histidine phosphotungstate was soluble to the extent of 28.6 mg. per liter.

The test is interfered with by a large excess of both cystine and arginine.

Jorpes (346) found that the Koessler-Hanke directions gave unsatisfactory results, that the color intensity was greatly influenced by the conditions of the reaction, and that the test is non-specific, being given by tyrosine, phenols, guanine, adenine, imidazoles, sulfides, ammonium ions, etc.

C. Jorpes' Modification of the Diazo Test (346)

Method: To 1 ml. of histidine solution (0.005 to 0.05 mg. of histidine) add 2 ml. of Weiss-Ssobolew diazo reagent, wait 1 to 3

hours, then add 5 ml. of 1.1 per cent Na_2CO_3. Read in 4 to 8 minutes with a 500 mu filter.

D. Lang's Modification of the Pauly-Weiss Method (401)

Principle: Histidine is separated from tyrosine by quantitative precipitation with $HgCl_2$.

Reagents: Mercury Chloride. To 150 ml. saturated solution of $HgCl_2$ add 70 gm. of sodium acetate and 10 gm. of NaCl (Hinsberg and Laszlo reagent).

Method: 1. Precipitation of Histidine. Neutralize 1 ml. of an HCl protein hydrolysate, containing 30 to 40 mg. of protein with saturated Na_2CO_3, add 1 drop of N HCl, 1 ml. of $HgCl_2$ reagent and 2 ml. of 4 per cent $NaBO_2$, mix, stand some time. Centrifuge and wash the precipitate.

2. Estimation of Histidine. Dissolve the histidine mercury complex in 2 or 3 drops of 5 per cent NaCN and transfer the solution to a 50 ml. volumetric flask. Add 2 ml. of saturated Na_2CO_3 and 4 ml. of freshly prepared p-diazobenzene sulfonic acid reagent. Read using filter 530 mu.

E. Macpherson's Modification of the Pauly Reaction (434)

Principle: The sulfanilic acid is diazotized in the presence of histidine rather than separately.

Method: To 0.01 to 0.20 mg. of histidine in 10 ml. of H_2O add 1 ml. of 1 per cent sulfanilic acid in 10 per cent by volume of HCl, 1 ml. of 5 per cent $NaNO_2$, mix and stand at room temperature for 30 minutes. Add 3 ml. of 30 per cent Na_2CO_3, mix, add 10 ml. of alcohol, cool under the tap and dilute to 25 ml. with water. Read with 530 mu filter.

Comment: This appears to be the best modification of the Pauly test. The histidine is separated from tyrosine and other substances by phosphotungstic acid (630, 639), $AgNO_3$-$Ba(OH)_2$ (379, 86), $HgCl_2$-Na_2CO_3 (378, 405, 242), etc.

2. THE REACTION OF HISTIDINE WITH BROMINE (KNOOP'S TEST)

Historical: Knoop reported in 1908 (372) that when an aqueous solution of histidine or its salts is treated with a slight excess of bromine water and the solution is heated, it becomes first colorless, then reddish and finally dark wine red. After a while a dark amorphous precipitate appears. If the solution is made alkaline the color remains and the precipitate does not form. Only imidazolethylamine of numerous histidine derivatives tried gave the color. The test is sensitive to 1 part per thousand.

A. Hunter's Modification of the Knoop Test

Hunter (311) confirmed Knoop's finding that an excess of bromine during the heating was harmful and removed it by $CHCl_3$ extraction. After bromination, the addition of ammonia gave a deep purple color.

B. Kapeller-Adler's Adaptation of the Knoop-Hunter Reaction (351)

Reagents: Bromine. 1 per cent by volume of bromine in 33 per cent acetic acid.

Ammonia. 2 parts of concentrated NH_4OH plus one part of 10 per cent $(NH_4)_2CO_3$.

Method: 1. Hydrolysis. Two to 3 gm. of protein are hydrolyzed with 25 per cent H_2SO_4 for 20 hours. The excess acid is removed with $Ba(OH)_2$. The filtrate and washings are concentrated to a convenient volume. A nitrogen determination on an aliquot of the hydrolysate is used to give the concentration of protein!

2. Separation of Histidine. An aliquot of the above, equivalent to 500 to 1000 mg. of protein, is evaporated to a small volume. Sufficient alcohol is added to incipient precipitation. Then one third the volume of ether is added and an excess of 10 per cent $HgSO_4$ in 5 per cent H_2SO_4 (Denigès' reagent). After standing over night, the histidine mercury complex is filtered and washed with alcohol and ether. The precipitate is decomposed with H_2O-HCl-H_2S. The filtrate is evaporated to dryness and dissolved in 10 per cent H_2SO_4.

3. Destruction of Tyrosine. To 1 or 2 ml. of the histidine solution (1 or 2 mg. of histidine), $N/10$ $KMnO_4$ is added drop by drop to a faint pink. The solution is warmed gently to dissolve any MnO_2.

4. Bromination. Bromine reagent is added drop by drop until a permanent yellow color remains for 10 minutes. If it fades, a few drops more of bromine reagent are added.

5. Development of Color. 2 ml. of ammonia reagent are added and the solution is placed in boiling water for 5 minutes, cooled and diluted with ammonia reagent to 10 ml. The deep purple color is read with filter 500 mu (352).

Comments: 1. Purification of Histidine. Woolley and Peterson (691) separate the histidine from the other components in the protein hydrolysate by precipitation first with $AgNO_3$-$Ba(OH)_2$ or $HgSO_4$ in H_2SO_4 and then with phospho-24-tungstic acid (cf. Kossel procedures above).

Block (95) recommends carrying out the test on the histidine silver filtrate after removal of the excess reagents.

2. Bromination. The most delicate part of the Knoop-Hunter

test is the quantity of bromine to add. If either too much or too little is introduced the results are erratic and low. Hunter (311) removed the excess bromine with chloroform. Földes (227) added varying quantities of bromine reagent until there was an apparent excess which was confirmed with a few drops of starch-KI solution. If blue, 0.2 ml. less of reagent were added to a new solution.

Woolley and Peterson (691) added an excess of bromine to the histidine solution, the excess is then removed by aeration before adding the NH_4OH.

Conrad and Berg (168) destroyed the excess bromine after 10 minutes at room temperature by a drop of saturated As_2O_3 in 10 per cent ammonia, while Langley (402) used 0.5 per cent phenol for the same purpose.

3. Development of Color. Racker (536) has abandoned Hunter's use of ammonia. He added dry Na_2CO_3 until the production of CO_2 ceased in the warm solution. The color is read after the tube has stood 10 minutes at room temperature.

Langley (402) introduced the bromine by aspirating in 1.5 ml. of a 1 per cent solution of Br_2 in $CHCl_3$. After removing the excess bromine with 0.5 per cent phenol, 1 ml. of saturated sodium acetate solution is added and the color is developed at pH 4.5, by heating in a boiling water bath for 1 minute in the dark. It is then cooled and diluted to 10 ml. with water.

C. Plimmer and Phillips' Modification of the Knoop Reaction (519)

Principle: Standard bromine as KBr-$NaBrO_3$ is added to a tyrosine-free histidine solution. The excess Br_2 is titrated with KI-$Na_2S_2O_3$. The results are erratic.

PART IV

DIRECT DETERMINATION OF LYSINE

1. THE NINHYDRIN REACTION (RUHEMANN-VAN SLYKE)

Historical: In 1912, Ruhemann (559) reported that when free amino acids are warmed in faintly acid or neutral solution with triketohydrindene hydrate (ninhydrin), they are decomposed to yield CO_2, NH_3 and the next lower aldehyde. Glycine is an exception to this rule and does not yield HCHO.

Ninhydrin
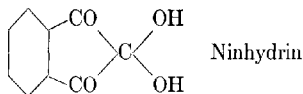
A. The Procedure of Van Slyke, Dillon, MacFadyen, and Hamilton, (636, 637, 638)

Principle: Lysine is precipitated by phospho-24-tungstic acid. After removal of the reagents, the quantity of lysine can be calculated from the formula:

Lysine N = $2 \times$ (Amino Nitrogen-Carboxyl Nitrogen)
where Amino N is determined by the reaction with HONO
and Carboxyl N is determined by the reaction with ninhydrin

Apparatus: Carbon dioxide can be determined either in the well-known Van Slyke-Neill manometric gas analysis apparatus or more conveniently (638) in two 25 ml. Erlenmeyer flasks (one for the reaction, one for standard $Ba(OH)_2$) without lips, which are connected to each other by means of a U-tube. The U-tube has a small side-arm which permits the entire apparatus to be evacuated (638).

Reagents: Citrate buffers: *p*H 4.7: grind together 17.65 gm. $Na_3C_6H_5O_7 \cdot 2H_2O$ and 8.40 gm. $C_6H_8O_7 \cdot H_2O$ to a fine powder.

:*p*H 2.5: grind together 2.06 gm. $Na_3C_6H_5O_7 \cdot 2H_2O$ and 19.15 gm. $C_6H_8O_7 \cdot H_2O$.

Phosphoric Acid: *p*H 1: mix 1 volume of H_3PO_4 (sp. gr. 1.72) with 1.5 volumes of H_2O. Titrate an aliquot of this dilute acid and adjust so that the strength is 6.0 ± 0.1 M.

Standard $Ba(OH)_2$: 0.25 N: cold saturated $Ba(OH)_2$ is adjusted to 0.3 N with CO_2-free water. 5 volumes of this solution are mixed

48

with 1 volume of 12 per cent $BaCl_2$ prepared, of course, in CO_2-free H_2O. The standard baryta should be titrated with $N/7$ HCl to pH 8 with 1 drop of 1 per cent phenolphthalein as the indicator.

Veronal buffer: pH 8: 10.3 gm. sodium veronal in 500 ml. of water. Mix 7 ml. of this solution with 4 ml. of $N/14$ HCl, add phenolphthalein. This is the color to which the $Ba(OH)_2$ is titrated.

Method: 1. Reaction of Hydrolysate. Adjust to faintly acid to brom phenol blue.

2. Removal of Preformed CO_2. To 1 to 5 ml. of solution in the 25 ml. Erlenmeyer flask add 50 to 100 mg. of either pH 2.5 or 4.7 citrate buffer and 1 drop of caprylic alcohol. Boil off preformed CO_2. Stopper and cool to 15°. In the meantime remove CO_2 from titration flask by a stream of CO_2-free air. Add 3 ml. of 0.25 N-$Ba(OH)_2$ to the titration flask.

3. Oxidation. Add 50 to 100 mg. of ninhydrin to reaction flask. Connect to $Ba(OH)_2$ flask by means of U-tube and rubber tubing. Make connections glass to glass. Evacuate at the water pump and immerse entire apparatus as far as the top of the U-tube in boiling water for 7 minutes or longer.

4. Distillation of CO_2. Cool the $Ba(OH)_2$ flask in water but keep the reaction vessel at 100°. Shake for 3 minutes to facilitate the distillation and absorption of all CO_2.

5. Titration. Titrate $Ba(OH)_2$ with $N/7$ HCl from a 5 ml. burette using 1 drop of 1 per cent phenolphthalein as the indicator. Subtract reagent blank.

$$1 \text{ ml. } N/7 \text{ HCl} \approx 1 \text{ mg. of carboxyl nitrogen}$$

Comment: As far as we know, this method has not yet been used to determine lysine in protein hydrolysates.

Van Slyke *et al.* (637) point out that isatin,
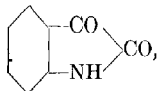
in glacial acetic acid and chloramine T, $CH_3C_6H_4SO_2NClNa$, at pH 2.5 and up may be used in place of ninhydrin. The original papers should be consulted for experimental details.

Christensen, West, and Dimick (161) find that lysine yields 112.5 per cent of the expected CO_2 with ninhydrin at faintly acid reaction (KH_2PO_4 buffer).

2. THE LIBERATION OF FREE AMINO GROUPS (VAN SLYKE-LIEBEN)

Historical: Since Van Slyke showed in 1911 (628) that alpha amino groups react more rapidly with nitrous acid than does the

epsilon amino group of lysine and since numerous investigators in-
cluding Van Slyke and Birchard (632) pointed out the close con-
nection between free amino groups in intact proteins and their
lysine content, many efforts have been made to utilize these ob-
servations for a simple method of determining lysine. The recent
experiments of Lieben and Loo (420) appear to have achieved this
goal.

A. The Liberation of Amino Nitrogen from Intact Proteins
(Lieben, 420)

Apparatus: The standard Van Slyke amino nitrogen apparatus
with wide capillaries, to avoid obstruction by particles of protein,
is employed.

Method: 60 to 80 mg. of protein are stirred in a beaker with a few
drops of water and then quickly dissolved in glacial acetic acid. The
solution is washed into the Van Slyke apparatus which already
contains glacial acetic acid, $NaNO_2$, caprylic alcohol, etc. Readings
are taken at 30, 60, and 90 minutes.

Calculation:

Σ-Amino N $= \Sigma = C - 3(B - A)$ where

A is mg. of amino N liberated in 30 minutes per 100 mg. of protein
B is mg. of amino N liberated in 60 minutes per 100 mg. of protein
C is mg. of amino N liberated in 90 minutes per 100 mg. of protein

Lysine $= 146/14 \times \Sigma$.

PART V

DETERMINATION OF HYDROXYLYSINE

Historical: The consistently higher values for "lysine" found by the Van Slyke nitrous acid method compared to those found by the Kossel isolation procedures (*cf.* Osborne, Van Slyke, *et al.* 505), suggested to Van Slyke the possibility that another basic amino acid was present in the phosphotungstate precipitate. This idea was substantiated by the isolation of hydroxylysine by Schryver, Buston, and Mukherjee (570) in 1925.

A. Schryver's Isolation of Hydroxylysine by the Carbamate Method

This is probably not a quantitative procedure and will only be described in substance.

Method: 1. The protein is hydrolyzed with H_2SO_4.

2. The amino acids are precipitated in alcoholic solution as the barium carbamates at 0° with $Ba(OH)_2$ and CO_2.

3. Glycine and hydroxylysine barium carbamates are insoluble in ice water. These are separated from the others and are decomposed by boiling water. Any $Ba(OH)_2$ is removed with CO_2.

4. Hydroxylysine is precipitated with phosphotungstic acid in 5 per cent H_2SO_4 and further purified by precipitation with $HgCl_2$ and $Ba(OH)_2$ to slight alkaline reaction. The hydroxylysine was isolated as the picrate from the $HgCl_2$ precipitate after removal of the reagents.

Comment: This work has not been generally accepted.

*B. Determination of Hydroxylysine by Periodate Oxidation (639)

Principle: Hydroxylysine is precipitated with 5 to 8.5 per cent phosphotungstic acid in 0.25 N HCl. Hydroxylysine liberates 1 mol of ammonia when treated in alkaline solution with periodic acid (Van Slyke, Hiller, and MacFadyen (639).

Method: 1. Hydrolysis. 3 gm. of protein are refluxed for 24 hours with 9 ml. of 6 N HCl. The excess acid is removed by concentration *in vacuo* and the ammonia and humin by $Ca(OH)_2$ as usual.

2. Precipitation with Phosphotungstic Acid. The ammonia-free protein hydrolysate is diluted to 300 ml., neutralized, and 6 ml. of concentrated HCl are added (i.e., approximately 0.25 N HCl). 25 gm. of phospho-24-tungstic acid in a small quantity of 0.25 N HCl

51

are added to the hot solution and the precipitate is allowed to form at room temperature (23 to 25°) for 48 hours. The precipitate is washed 5 times with 8 ml. portions of 5 per cent phosphotungstic acid in 0.25 N HCl.

3. Reprecipitation with Phosphotungstic Acid. The washed precipitate is dissolved by the aid of 2 N NaOH until the solution is neutral to alizarin red. The solution is diluted to 300 ml., 6 ml. of concentrated HCl are added, the solution is heated and 15 gm. of phosphotungstic acid in a little 0.25 N HCl are added. After standing at room temperature for 48 hours, the precipitate is washed with phosphotungstic acid, and dissolved in NaOH as before.

4. Oxidation. To 5 ml. of the above filtrate, 1 ml. of 5 per cent glycine, 1 drop of caprylic alcohol, 1 ml. of 2 N NaOH, 2 ml. of 4.6 per cent (0.2 M) $HIO_4 \cdot 2H_2O$, and 10 ml. of saturated K_2CO_3 are added. The NH_3 is aerated for 25 minutes into 2 per cent boric acid.

5. Determination of Hydroxylysine. The ammonia is titrated with standard HCl using methyl red-methylene blue as the indicator.

Comment: Van Slyke, Hiller, and MacFadyen (639) find that the diamino acids are precipitated as mixed salts and not as individual substances each with its own individual solubility. The effect on a given diamino acid in small amount or forming a more soluble phosphotungstate than the average of the group, is to diminish the loss of such an amino acid below the loss that would be calculated from the solubility of its isolated phosphotungstate. One cannot apply a simple solubility correction to hydroxylysine phosphotungstate, but if the total diamino nitrogen is less than 10 per cent of the total nitrogen, then it is advisable to add sufficient arginine or lysine to bring the same to 15 or 20 per cent of the total N.

It should be noted that the presence of sodium phosphotungstate does not interfere with the oxidation of hydroxylysine or with the distillation of the ammonia.

CHAPTER I

PART VI

DETERMINATION OF CITRULLINE

A. Fearon's Diacetyl Method (217)

Principle: Diacetyl, $CH_3COCOCH_3$, in strong acid solution does not give a color with arginine and other guanidine derivatives (*cf.* Harden-Norris reaction above) but does with citrulline and substituted ureas (217).

Citrulline $NH_2CONHCH_2CH_2CH_2CHNH_2COOH$

Method: A small quantity of protein is dissolved in 2 ml. of dilute HCl, 4 ml. of concentrated HCl and 3 to 5 drops of 3 per cent aqueous diacetyl monoxime are added. The solution is boiled for 30 seconds, cooled 2 minutes, and 1 to 3 drops of 1 per cent $K_2S_2O_8$ are added. A carmine color is developed especially on gentle warming.

Comment: As tryptophane plus carbohydrate will give a Molisch reaction under these conditions, one or the other will have to be absent.

Sensitivity 0.01 to 0.2 per cent citrulline.

B. Gornall and Hunter's Modification of the Fearon Test (262)

Method: To 7 ml. of unknown (0.05 to 0.07 mg. of citrulline) add 4 ml. of concentrated HCl (sp. gr. 1.18–1.19) and 0.5 ml. of 3 per cent diacetylmonoxime. Place in a gently boiling water bath to the level of the liquid in the tubes for 9 minutes. Use funnels as condensers. *Remove tubes and cool for 6 minutes. Add 1 drop of 1 per cent $K_2S_2O_8$* and read the maximum color in an Evelyn type colorimeter with filter 490 mu. When the galvanometer becomes stationary add a second drop of $K_2S_2O_8$. If this produces a further increase in color add a 3rd drop.

Comment: The intensity of the color is a function of the concentration of all reagents, time of heating, etc.

Urea, allantoine, substituted ureas, etc. interfere.

PART VII

DIAMINO ACIDS IN PROTEINS

All values given in the following tables have been calculated to 16.0 per cent of nitrogen. In those cases, where nitrogen values are not given by the authors, the amino acid values were recalculated using a value of N which is given in parenthesis. If the investigator reported the data in amino acid nitrogen as per cent of total nitrogen, then the results have been recalculated to 16.0 per cent of nitrogen, but no value for N is given in the tables. The data in the tables can thus be easily recalculated to amino acid N in per cent of total N if the reader wishes.

Although this procedure may have introduced errors in certain cases, it is believed that the advantages outweigh the disadvantages especially when the figures are to be used for comparative purposes in nutritional investigations.

Our own published and unpublished results have been recalculated to 16.0 per cent of N and corrected, wherever justified, for the following "overall losses":

If the N of the preparation analyzed was *below* 12 per cent, then 28 mg. of arginine, 12 mg. of histidine, and 28 mg. of lysine were added to the quantities actually isolated.

If the N of the preparation analyzed was above 12 per cent, then corrections of 18 mg. of arginine, 9 mg. of histidine, and 14 mg. of lysine were used.

As we have not determined the "overall losses" for quantities of protein over 2.5 gm., the above corrections were used only when 2.5 gm. or less of protein were hydrolyzed. If more than 2.5 gm. of protein were analyzed, only the arginine was corrected by the Gulewitsch factor (270).

The data in the tables are self explanatory, but a few brief comments may be in order. Those figures designated "Best Values" are indicated purely for the reader's convenience and represent only the authors' personal opinions. The mean values have been calculated with twice the standard error (2×S.E.) as follows:

$$\text{S.E.} = \frac{\text{S.D.}}{\sqrt{N}}$$

$$\text{S.D.} = \sqrt{\frac{\Sigma x^2 - \bar{x}^2}{N - 1}}$$

where

$\Sigma x^2 =$ the sum of the squares of the observations

$N =$ the number of observations

$\bar{x}^2 =$ the square of the mean

S.D. = the standard deviation

S.E. = the standard error.

In a few instances, values which were added to the tables after the calculation of the means were not included therein for obvious reasons.

ALBUMINOIDS

Basic Amino Acids in *Gelatin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
			per cent	gm.	gm.	gm.	
Electrolytic-Kossel	Albanese	27	16.1	5.7	1.2	3.6	Lysine from N only
Electrolytic-Kossel	Albanese	27	15.2	5.8	0.7	5.3	Lysine from N only
Kossel-Block	unpublished		14.7	8.2	0.7		Difco
Kossel-Block	unpublished		16.6	7.2	0.8	4.3	Pork Skin-Wilson
Kossel-Block	unpublished		16.0	7.9	1.0	4.5	Bone-Wilson
Kossel-Block	unpublished		15.4	7.7	0.8	4.3	Coignet
Van Slyke	Dakin	185	18.0	7.3	0.8	5.2	
Kossel-Fürth	Fürth	251	17.0	9.1			
Kossel-Gross	Fürth	251	17.0	7.7			
Jansen-Graff	Graff	263		7.8			
Pauly-Koessler	Hanke	280	(16.0)		0.5		
Kossel-Kutscher	Hart	283	16.0	7.6	0.4	2.8	
Jansen-Hunter	Hunter	313		7.8			
Jansen-Hunter	Hunter	314		7.8			
Kossel-Kutscher	Kossel	379	(16.0)	9.3			from N only
Kossel-Gross	Kossel	384		8.2			
Van Slyke	Narayana	471		7.9	1.6	3.4	
Sakaguchi	Sakaguchi	563		7.6			
Van Slyke	Van Slyke	631		7.3	2.6	5.3	
Van Slyke	Van Slyke	639					0.7 to 0.8 per cent HO·lysine
Kossel-Vickery	Vickery	656	18.3	7.6			direct method
Lieben-Loo	Lieben	420	15.9			4.7	
"Best Values"			18.0	8.0	0.8	4.5	
Mean				7.6±0.1	1.0±0.1	4.3±0.2	with 2×S.E.

ALBUMINOIDS

Basic Amino Acids in *Elastins, Collagens, and Related Proteins*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Elastin	Kossel-Block	Stein	586	17.1	0.9	0.0	?	
Lens	Kossel-Kutscher	Hijikata	298	(16.0)	3.3	1.6	1.6	
Neurogelatin	Kossel-Block	unpublished		14.7	7.8	1.5	5.4	
Fish gelatin	Kossel-Block	unpublished		11.8	5.4	2.6	4.1	"Stick"
Fish gelatin	Schryver	Schryver	570		3.1 per cent hydroxylysine			

COMMENTS—ALBUMINOIDS

Gelatin: The variations in the basic amino acids in the gelatins
are due, in part, to actual differences in composition and probably
not entirely to experimental errors.

Elastin: This protein is almost completely lacking in the basic
amino acids.

Fish gelatin or "Stick water" appears to be richer in histidine
than the common animal gelatins. It is probably even more hetero-
geneous in composition than gelatin.

ANIMAL PROTEINS

Diamino Acids in *Entire Animals*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Rat	Kossel-Block	Block	87	15.2	6.1		5.2	
Rat	Kossel-Block	unpublished		13.3	6.2	2.8	6.1	1 to 2 days old
Rat	Kossel-Block	unpublished		12.2	7.1	2.3	6.5	23 days old
Rat	Kossel-Block	unpublished		13.4	7.1	1.8	5.6	100 days old
Rat	Kossel-Block	unpublished		12.2	7.3	1.7	6.5	540 days old
Guinea pig	Kossel-Block	Block	87	12.6	6.7		5.6	
Chicken	Van Slyke	Patton	512		5*	4*	9*	
Chicken	Kossel-Calvery	Calvery	141	(15.0)	5.8	1.4	6.6	Embryo
"Best Values"				16.0	7.0	2.2	6.2	
Mean with 2 ×S.E.					6.6±0.4	2.0	6.0±0.3	

* Omitted from Mean.

COMMENTS ON ANIMAL PROTEINS

The analysis of whole animals indicates that they contain ap-
proximately 7 per cent of arginine, over 2 per cent of histidine, and
6 per cent of lysine. Therefore, it is logical to assume that for the
rapidly growing animal, the protein mixture to be fed should con-
tain these three amino acids in approximately this proportion. This
is especially true in the case of histidine and lysine as these do not
appear to be synthesized by mammals even at a slow rate.

BLOOD PROTEINS

Diamino Acids in *Fibrin*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Beef	Kossel-Vickery	Vickery	656	16.8	7.8			direct
Beef	Pauly-Koessler	Hanke	280	(17.5)		1.9		
Beef	Sakaguchi, Pauly	Jorpes	344	16.8.	7.6	2.1		
Beef	Knoop-Kapeller	Kapeller-Adler	351	(17.5)		3.2		
Beef(?)	Jansen-Hunter	Hunter	313		7.1			
Beef(?)	Sakaguchi	Sakaguchi	563		6.9			
Beef(?)	Van Slyke	Van Slyke	630		6.9	2.8	9.7	
Beef	Electrolytic-Kossel	Albanese	27	15.2	7.0	2.2	6.0	Lysine by N.
Beef	Kossel-Vickery	Bergmann	66	17.7	6.7	2.3	9.0	corrected
Beef	Kossel-Fürth	Fürth	251	(17.5)	6.3			
Sheep	Pauly-Koessler	Hanke	280	(17.5)		2.0		
Swine	Pauly-Koessler	Hanke	280	(17.5)		2.1		
Horse	Kossel-Kutscher	Lock	422		4.7	2.2	5.4	
"Best Values"				17.0	7.8	2.4		
Mean with 2×S.E.					6.8±0.6	2.3±0.2	7.5	

BLOOD PROTEINS

Diamino Acids in *Hemoglobins*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Horse	Kossel-Block	Block	84	16.7	3.5	7.7	8.2	
Horse	Electrolytic-Kossel	Albanese	27	13.4	3.5	8.5	5.9	Lysine by N.
Horse	Kossel-Vickery	Vickery	645	16.7	3.2	7.3	7.7	
Horse	Kossel-Vickery	Vickery	656	16.7	3.4			direct method
Horse	Kossel-Vickery	Vickery	659	16.7	3.4	7.3		direct method
Horse	Sakaguchi, Pauly	Jorpes	344	16.8	3.2	7.4		
Horse	Knoop-Kapeller	Kapeller-Adler	351	(16.8)		7.1		
Horse	Kossel-Fürth	Fürth	251	(16.8)	4.2			
Horse	Pauly-Koessler	Hanke	278	(16.8)		8.4		
Horse	Van Slyke	Hunter	312	16.9	3.9	7.3	9.1	
"Best Values"				16.7	3.5	7.4		
Mean with 2×S.E.					3.5±0.3	7.6±0.4	7.7	
Sheep	Kossel-Vickery	Vickery	659	16.9	3.7	7.0*		direct method
Sheep	Kossel-Block	Block	84	16.8	3.9*	7.3	7.9	
Sheep	Pauly-Koessler	Hanke	278	(16.8)		8.4		
Beef	Kossel-Vickery	Bergmann	67	17.0	2.9	7.0	7.5*	
Beef	Pauly-Koessler	Hanke	278	(16.8)		7.5*		
Beef	Van Slyke	Van Slyke	630		3.8	7.5	9.2	
Dog	Kossel Block	Block	84	16.4	4.0	7.4	8.5	
Cat	Pauly-Koessler	Hanke	278	(16.5)		8.3		
Human	Kossel-Vickery	Vickery	659	17.0	4.0	7.6		direct method
Turtle	Kossel-Block	unpublished		15.5	2.8	4.6		

* "Best Values."

BLOOD PROTEINS
Diamino Acids in *Globins*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Beef	Van Slyke, Pauly	Roche	550	16.6	3.3	7.5	8.2	Sakaguchi
Beef	Sakaguchi-Dumazert	Dumazert	200		3.6			
Horse	Van Slyke, Pauly	Roche	550	16.8	3.4	7.6	7.6	Sakaguchi
Horse(?)	Jansen-Hunter	Hunter	313		3.8			
Dog	Van Slyke, Pauly	Roche	550	16.6	3.3	8.0	8.0	Sakaguchi
Guinea Pig	Van Slyke, Pauly	Roche	550	16.7	3.3	8.5	8.9	Sakaguchi
Human	Van Slyke, Pauly	Roche	550	16.7	3.3	8.2	8.8	Sakaguchi
Human	Kossel-Block	unpublished		16.2	3.4	7.4		
Rabbit	Van Slyke, Pauly	Roche	550	16.6	3.3	8.3	8.4	Sakaguchi
Sheep	Van Slyke, Pauly	Roche	550	16.8	3.4	7.6	8.0	Sakaguchi
Sheep	Van Slyke, Pauly	Roche	550	16.3	3.4	7.7	8.5	Sakaguchi
"Best Values"				16.7	3.4	8.0	8.0	
Mean with 2×S.E.					3.4 ± 0.1	7.9 ± 0.3	8.3 ± 0.3	

BLOOD PROTEINS
Diamino Acids in *Serum Albumins*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Beef	Kossel-Block	unpublished		15.7	5.7	3.0	7.6	1:4 3M PO₄ buffer
Beef	Kossel-Block	unpublished		14.7	5.3	3.0	7.2	½ sat. (NH₄)₂SO₄
Beef	Kossel-Block	Block	81	14.4	5.2	2.4	10.4	½ sat. (NH₄)₂SO₄
Beef	Kossel-Block	Block	81	14.4	5.7	2.4	10.3	½ sat. (NH₄)₂SO₄
Beef	Sakaguchi	Sakaguchi	563		5.1			
Human	Kossel-Block	Murrill	469	13.5	5.3	2.6	10.4	
Human	Harden-Lang	Lang	399	(16.0)	4.1			
Human	Pauly-Lang	Lang	401			2.8		
Human	Van Slyke	Cavett	153		6.2		14.6	
Human	Sakaguchi	Brand	127	(16.0)	6.3			
Horse	Kossel-Kutscher	Lock	422		4.6	2.6	8.3	Crystalline
Horse	Sakaguchi	Brand	127	(16.0)	5.5			
"Best Values"				15.8	5.5	2.8	10.0	
Mean with 2×S.E.					5.2 ± 0.4	2.7 ± 0.2	9.8 ± 1.9	

BLOOD PROTEINS

Diamino Acids in *Serum Globulins*

Calculated to 16 0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Beef	Kossel-Block	unpublished		14.2	5.3	2.3 •	6.1	1:4 3m PO₄ buffer
Beef	Kossel-Block	unpublished		14.1	5.0	2.7	5.8	½ sat. (NH₄)₂SO₄
Beef	Kossel-Block	Block	81	14.2	5.6		7.0	¼ sat. (NH₄)₂SO₄
Beef	Kossel-Block	Block	81	14.2	5.8		6.9	½ sat. (NH₄)₂SO₄
Beef	Sakaguchi	Sakaguchi	563		5.0			
Human	Kossel-Block	Murrill	469	14.0	4.7	2.3	7.0	
Human	Harden-Lang	Lang	399	(16.0)	4.2			
Human	Van Slyke	Cavett	153	16.0	5.4	1.4	10.9	pseudo
Horse	Sakaguchi, Pauly	Jorpes	344	15.5	5.7	2.2		
Horse	Kossel-Kutscher	Lock	422		3.8	1.4	5.2	
Horse	Kossel-Calvery	Calvery	144	16.0	5.7	1.1	4.9	Pneumococcus-
Horse	Kossel-Calvery	Calvery	145	(16.0)	5.0	1.0	5.4	precipitates
Dog	Sakaguchi-Thomas	Thomas	607	(16.0)	6.4			
"Best Values"				16.0	5.3	2.4	6.5	
Mean with 2 ×S.E.					5.2 ± 0.4	1.8 ± 0.5	6.6 ± 1.2	

BLOOD PROTEINS

Diamino Acids in *Human Serum Proteins*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE
			per cent	gm.	gm.	gm.
Kossel-Block	Block	105	14.7	5.0	2.3	6.7
Kossel-Block	Block	105	15.9	5.1	2.5	6.7
Kossel-Block	Block	105	15.0	5.8	3.1	7.3
Kossel-Block	Block	105	15.0	5.5	2.6	7.3
Kossel-Block	Block	102	15.2	5.7	2.5	7.6
Kossel-Block	Block	102	15.2	5.4	2.6	7.1
Kossel-Block	Block	102	15.0	5.5	2.7	6.8
Kossel-Block	Block	102	14.9	5.1	2.6	7.4
Kossel-Block	Block	102	14.3	5.5	3.1	7.6
Kossel-Block	Block	102	14.9	5.3	2.8	9.0
Kossel-Block	Block	102	14.9	5.9	2.6	6.9
Kossel-Block	Block	102	14.9	5.6	2.7	7.1
Kossel-Block	Block	82	14.1	5.6		7.6
Kossel-Block	Block	82	14.1	5.8		8.4
Kossel-Block	Murrill	469	14.3	5.2	2.5	9.6
"Best Values"			15.2	5.6	2.6	
Mean with 2 ×S.E.				5.5 ± 0.3	2.6 ± 0.1	7.5 ± 0.5

BLOOD PROTEINS

Diamino Acids in *Human Pathological Serum and Urine Proteins*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Serum	Kossel-Block	Block	82	15.2	5.5		6.4	Nephrosis
Serum	Kossel-Block	Murrill	469	14.7	5.2	2.5	9.6	Nephritic
Serum	Kossel-Block	unpublished		14.1	5.2	2.2	5.7	Myeloma
Urine	Kossel-Block	Block	82	14.6	5.5		8.1	Nephrosis
Urine	Kossel-Block	Murrill	469	15.1	5.6	2.5	10.4	Nephritic
Urine	Van Slyke	Cavett	153		6.0	0.9	13.9	Albumin
Urine	Van Slyke	Cavett	153		5.3	1.5	9.2	Globulin
Urine	Kossel-Block	Devine	195	14.7	3.9	1.0	4.4	Bence-Jones
Urine	Kossel-Calvery	Calvery	143	18.1	5.2	1.2	7.0	Bence-Jones
Urine	Kossel-Kutscher	Hopkins	308	16.2	6.0	0.8	3.7	Bence-Jones

BLOOD PROTEINS

Diamino Acids in *Dog Serum and Plasma*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE
			per cent	gm.	gm.	gm.
Kossel-Block	Murrill	470	12.2	5.8	1.8	9.1
Kossel-Block	Murrill	470	11.6	5.8	1.9	8.9
Kossell-Block	Murrill	470	14.4	5.9	1.9	8.2
Kossell-Block	Murrill	470	12.7	6.0	1.8	7.8
Kossel-Block	Murrill	470	12.2	6.0	1.8	8.2
Kossel-Block	Murrill	470	14.1	5.6	2.0	9.2
Kossel-Block	Murrill	470	14.2	5.8	1.9	8.1
Sakaguchi-Thomas	Thomas	607	(16.0)	5.6*		
Sakaguchi-Thomas	Thomas	607	(16.0)	4.5		
Kossel-Block	unpublished		14.5	6.1		8.6
Kossel-Block	unpublished		14.7	6.2		8.7
Kossel-Block	unpublished		14.6	6.6		8.6
"Best Values"			15.0	6.0	1.9	8.5
Mean with 2×S.E.				5.8±0.3	1.9±0.1	8.5±0.3

* Plasma.

BLOOD PROTEINS

Diamino Acids in *Serum Proteins Other Than Human and Dog*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	AR-GININE	HIS-TIDINE	LYSINE	
				per cent	gm.	gm.	gm.	
Horse	Sakaguchi, Pauly	Jorpes	344	15.5	5.7	2.2		
Hen	Kossel-Block	Block	83	15.4	6.4	2.0	5.8	
Turkey	Kossel-Block	Block	83	16.0	7.2	2.5	6.2	
Duck	Kossel-Block	Block	83	15.4	6.9	2.7	6.3	
Cow	Kossel-Block	Block	83	14.2	5.6		7.9	
Turtle	Kossel-Block	unpublished		13.9	5.1	2.9	3.2	

BLOOD PROTEINS

Diamino Acids in *Stroma and Cell Proteins*

Calculated to 16 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	AR-GININE	HIS-TIDINE	LYSINE	
				per cent	gm.	gm.	gm.	
Horse	Sakaguchi, Pauly	Jorpes	344	15.4	6.4	2.9		
Horse	Kossel-Block	Beach	55	12.9	6.1	2.4	4.7	
Beef	Kossel-Block	Erickson	213	13.7	5.8	2.0	5.3	Embryo
Beef	Kossel-Block	Beach	55	13.8	5.9	2.2	4.1	
Sheep	Kossel-Block	Beach	55	14.0	6.1	2.6	4.0	
Hog	Kossel-Block	Beach	55	13.1	5.6	2.6	4.3	
Human	Kossel-Block	Beach	55	13.0	5.9	2.6	4.7	
Human	Kossel-Block	Erickson	213	13.1	6.1	2.3	5.0	Polycythemia vera
Human	Kossel-Block	Erickson	213	13.7	4.0*	2.5*	3.6*	Polycythemia cardiac
"Best Values"				15.4	6.1	2.6	4.8	
Mean with 2×S.E.					6.0±0.2	2.4±0.2	4.6±0.4	
* Omitted from Mean								
Human	Kossel-Block	Block	105	16.3	3.2	6.3	6.9	Cells

COMMENTS—BLOOD PROTEINS

Fibrin: The lysine content of this protein appears to be high but is not known with any degree of accuracy. There may be small specie differences.

Hemoglobins and Globins: As is well known these proteins are uniquely high in histidine. They also are a good source of lysine. There are slight, though probably significant specie differences in composition except in the case of turtle hemoglobin which appears to have quite a different content of arginine and histidine.

Serum Albumins and Globulins: These are highly complex mixtures of proteins the exact composition of which will vary with the mode of preparation. Serum albumins are an excellent source of lysine and consequently in cases where new formation of seralbumin is desired, foods rich in lysine are indicated.

Serum Proteins: Changes in the amino acid composition of entire serum proteins (orosins) in health and disease rather than in the composition of the albumins and globulins should be the object of investigation. This procedure avoids the possibility of misinterpreting results of elaborate fractionation experiments. Although such experiments may yield chemically homogeneous protein fractions, these proteins are probably only artifacts.

Although the results on pathological sera are very meagre, they appear to show that nephritic and nephrotic serum proteins have essentially a normal basic amino acid composition but that the proteins in multiple myeloma are deficient in lysine.

Studies on Bence-Jones protein suggest that this protein may vary in diamino acid composition in different cases.

There may be true specie differences in the amino acid composition of the serum proteins, although like the animal hemoglobins, the differences are quantitative rather than qualitative except in avian and especially in turtle serum proteins which appear to differ rather widely in lysine.

Stroma Proteins: These have been studied in detail only by one group of investigators (55, 213) who have shown that their composition varies in health and disease.

* BRAIN PROTEINS

Diamino Acids in *Human Brain Proteins from Nonpsychotic and Psychotic Individuals*
(*cf.* 94, 89, 92, 90 and unpublished results)

Calculated to 16.0 per cent N.

CAUSE OF DEATH	NITROGEN	ARGININE	HISTIDINE	LYSINE	
	per cent	gm.	gm.	gm.	
Cerebral Hemorrhage	13.7	6.5	2.8	6.8	♂
Cerebral Hemorrhage	14.9	6.2	1.8	6.1	♂
Trauma	15.1	6.2	2.4	6.2	♂
Trauma	13.0	7.4	3.2	6.8	
Trauma	15.1	6.5	2.4	6.2	♂
Trauma	14.2	6.4	3.4	6.3	♂
Suicide	14.0	6.6	2.9	6.9	♂
Suicide	12.8	6.4	2.3	6.7	♂
Suicide	13.2	6.9	2.7	6.4	♂
Alcoholism	14.9	6.3	1.9	6.2	♂
Alcoholism	15.0	6.5	2.3	6.6	
Arteriosclerosis	13.4	6.8	2.2	6.4	
Diabetic coma	13.4	6.9	2.9	6.0	♀
Puerperal Septicemia	13.9	6.4	2.9	6.0	♀
Aneurism	12.7	6.6	2.8	6.4	♀
Coronary occlusion	13.6	7.2	3.0	6.4	
Mean with 2 ×S.E.		6.6±0.2	2.6±0.2	6.4±0.1	
Infection	14.3	6.8	2.5	6.6	Phenylpyruvic oligophrenia
Unknown	12.0	6.6	2.4	3.9	Krabbe's disease
Unknown	14.1	6.1*	1.5*	3.1*	Late Amaurotic Idiocy

* Highest values of four closely checking replicate experiments.

BRAIN PROTEINS

Diamino Acids in *Monkey Brains* (*cf.* 89, 92, 90, and unpublished results).

Calculated to 16.0 gm. N.

DESCRIPTION	NITROGEN	ARGININE	HISTIDINE	LYSINE
	per cent	gm.	gm.	gm.
Young male	12.8	7.4		6.3
Adult male	14.3	6.5	2.7*	6.9
Adult male	14.4	6.4	1.9*	6.7
Adult male	14.7	6.6	2.1*	6.3
Cerebellum, male	14.9	6.8	2.7	6.7
Cerebral Hemispheres, male	13.9	6.3	2.7	6.3
Mid-brain, male	14.6	6.9	2.5	5.1
Still-born, male	14.6		2.2	
Still-born, female	14.5	6.3	2.5	7.2
Adult female	14.2	6.1	2.4*	6.1
Adult female	14.2	6.2	2.3*	5.4
Adult female	14.0	6.7	3.2*	5.4
Adult female	14.8	6.5	2.6	6.2
Cerebellum, female	15.1	6.7	2.6	6.7
Mid-brain, female,	14.6	6.8	2.3	6.0

BRAIN PROTEINS

Diamino Acids in *Sheep, Rat, and Beef Brain Proteins*

(*cf.* 89, 92, 90, and unpublished results)

Calculated to 16.0 gm. N.

ANIMAL	NITROGEN	ARGININE	HISTIDINE	LYSINE	
	per cent	gm.	gm.	gm.	
Sheep	11.9	6.9	3.2	6.0	Cerebellum ♂
Sheep	14.7	6.6	2.5	6.7	Cerebellum ♂
Sheep	14.7	7.0	2.6	6.6	Cerebellum ♀
Sheep	15.3	6.4	2.4	6.2	Cerebral hemispheres ♀
Sheep	13.2	6.7	2.2	5.7	Cerebral hemispheres ♂
Sheep	13.6		2.5	6.0	Cerebral hemispheres ♂
Sheep	14.4	6.5	2.5	5.7	Midbrain ♂
Sheep	14.6	6.9	2.9	6.1	Midbrain ♂
Sheep	15.0	6.7	2.8	6.4	Midbrain ♀
Sheep	14.4	6.7	2.5	6.5	Entire brain ♀
Sheep	14.4	7.1	2.8	6.6	Entire brain ♂
Rat	14.3	6.5	2.3	5.6	1 to 2 days old
Rat	14.5	6.5	2.3	5.9	6 to 9 days old
Rat	13.1	6.8	2.1	6.7	
Rat	14.4	6.8	1.7	6.3	
Rat	15.5	5.7	3.1		
Rat	15.0	6.2	3.1	6.1	Hist. by Knoop ♀
Rat	14.7	6.6	2.7	6.3	Hist. by Knoop ♂
Beef	12.9	6.6	2.0	5.3	Hist. by diflavianate
Beef	13.6	6.4	2.9	5.8	
Beef	14.7	6.3	2.0	5.4	
Beef	14.3	6.8	2.5	6.7	♀
Beef	14.0	6.6	2.5	6.9	♂

BRAIN PROTEINS

Summary of Diamino Acids in *Human, Monkey, Sheep, Rat, Beef,*
Dog, Guinea Pig, and Rabbit Brains

Calculated to 16.0 gm. N.

ANIMAL	NITROGEN	ARGININE	HISTIDINE	LYSINE	
	per cent	gm.	gm.	gm.	
Human	13.9	6.6±0.2	2.6±0.2	6.4±0.1	Nonpsychotic, 16 cases
Human	12.0	6.6	2.4	3.9	Krabbe's disease, 1 case
Human	14.1	6.1	1.5	3.1	Amaurotic Idiocy, 1 case
Monkey	14.4	6.6±0.2	2.5±0.2	6.2±0.3	15 specimens
Sheep	14.2	6.8±0.1	2.6±0.2	6.2±0.2	11 specimens
Rat	14.5	6.4±0.3	2.5±0.4	5.9±0.5	7 specimens
Beef	13.9	6.5±0.2	2.4±0.3	5.9±0.6	5 specimens
Pig	15.4	6.6	2.7	6.5	2 specimens
Puppies	13.4	7.0	2.6	6.1	1 specimen
Guinea Pig	14.8	7.1	3.0	6.4	2 specimens
Rabbit	12.8	6.7	2.3	6.7	2 specimens

COMMENT ON BRAIN PROTEINS

The analytical results which are summarized in the tables have all been carried out in one laboratory although by three different persons. Therefore, the values reported have greater comparative than absolute value. However, it appears that the protein preparation from the *single* case of late infantile amaurotic idiocy is defi-

nitely subnormal in its yield of lysine. There does not appear to
be any significant difference in the basic amino acids yielded by
normal human, monkey, sheep, rat, and beef brain proteins on
acid hydrolysis.

The suggestion made previously (92) by one of us (R. J. B.) that
there may be a sex difference in the basic amino acids yielded by
primate brain proteins is no longer tenable as the result of many
further analyses.

EGG PROTEINS

Basic Amino Acids in *Crystalline Egg Albumin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE	
			per cent	gm.	gm.	gm.	
Kossel-Block	Bernhart	73	15.3	5.5	1.8	4.8	
Kossel-Calvery	Calvery	139	(15.5)	5.2	1.4	4.1	
Jansen-Hunter	Hunter	313	16.0	5.2			
Pauly-Koessler	Hanke	280	(15.5)		2.4		
Kossel-Patten	Osborne	494	15.5	5.1	1.8	3.9	
Kossel-Block	Pottinger	527	(15.5)	6.2	2.4	3.9	
Sakaguchi	Sakaguchi	563		6.3			
Kossel-Block	Tristram	619		6.1	1.6	5.1	corrected
Van Slyke	Van Slyke	639					0.08% HO · lysine
Kossel-Vickery	Vickery	651	15.8	5.7	1.5	5.0	
Kossel-Vickery	Vickery	656	15.5	5.8			direct method
Kossel-Chibnall	Chibnall	160	15.8	5.7	1.5	5.1	
"Best Values"			15.5	5.8	2.0	5.0	
Mean Values				5.7±0.3	1.8±0.3	4.5±0.5	with 2×S.E.

EGG PROTEINS

Basic Amino Acids in *Egg Proteins* other than Crystalline Albumin

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE
				per cent	gm.	gm.	gm.
Conalbumin	Kossel-Block	Block	83	15.0	6.4*	3.0	8.0*
Conalbumin	Kossel-Patten	Osborne	507	(16.1)	5.1	2.2*	6.4
Globulin	Kossel-Block	Block	83	14.4	6.6	1.6	5.7
Globulin	Kossel-Fürth	Fürth	251	(15.0)	4.4		
Vitellin	Kossel-Calvery	Calvery	140	15.0	8.4*	1.3	5.8*
Vitellin	Van Slyke	Calvery	140	15.0	8.6	1.0	9.3
Vitellin	Kossel-Patten	Osborne	495	16.3	7.3	1.9*	4.7
Vitellin	Sakaguchi	Sakaguchi	563	16.0	7.5		
Livitin	Kossel-Vickery	Jukes	347	14.8	5.1	1.2*	5.0
Livitin	Kossel-Vickery	Jukes	348	15.5	5.8*	1.0	5.2*
Egg White	Kossel-Block	Block	83	14.8	5.7		6.1
Egg White	Kossel-Calvery	Calvery	141	(15.0)	5.8*	2.2*	6.5*
Egg Yolk	Kossel-Block	unpublished		14.6	7.2	2.6*	5.0
Egg Yolk	Kossel-Calvery	Calvery	141	(15.0)	8.2*	1.4	5.5*
Whole Egg	Kossel-Block	unpublished		14.1	7.0	2.4	6.0

* "Best Values."

COMMENTS—EGG PROTEINS

Crystalline Egg Albumin and Egg White. The composition of crystalline ovalbumin, the principal protein in egg white, is known with considerable accuracy. As far as the authors are aware there have been no comparative studies on the diamino acid composition of hens' and other crystalline egg albumins. Egg white appears to yield more lysine than crystalline albumin; this, however, may be due to technical variations and not be significant.

Yolk Proteins. These appear to be richer in arginine than the proteins of the white.

FOODS

Diamino Acids in *Feeds* and *Foods*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITRO-GEN	AR-GININE	HIS-TIDINE	LYSINE	
			per cent	gm.	gm.	gm.	
Bread	Kossel-Block	unpublished	11.2	3.5	2.3	2.8	Milk, yeast
Flour	Kossel-Block	unpublished	13.5	3.9	2.2	1.9	
Cereal	Kossel-Block	unpublished		5.0	1.7	3.0	"Wheatena"
Cereal	Kossel-Block	unpublished		5.4	2.7	3.4	"Ralston"
Cereal	Kossel-Block	unpublished	12.5	3.3	1.8	1.8	"Cream Farina"
Cereal	Kossel-Block	unpublished	13.6	3.0	1.6	1.6	"Cream of Wheat"
Cereal	Kossel-Block	unpublished	13.8	5.2	3.1	1.3	"New Cream of Wheat"
Cereal	Kossel-Block	unpublished		2.2	2.2	0.9	"Puffed Sparkies"
Cereal	Kossel-Block	unpublished		2.3	1.2	1.4	Cerevim
Corn Gluten Meal	Kossel-Block	unpublished	13.4	3.2	1.9	2.0	
Linseed Meal	Kossel-Block	unpublished		6.2	1.5	2.5	
Alfalfa Meal	Kossel-Block	unpublished	10.6	4.3	2.1	4.2	
Soybean Meal	Kossel-Block	unpublished		5.8	2.3	5.4	
Flaxseed Meal	Kossel-Block	unpublished		6.9	1.9	1.0	
Soybean Meal?		Heinrich 286	?	11.8	3.8	4.4	
Lupine Meal?		Heinrich 286	?	12.6	2.9	3.1	
Meat Scraps	Kossel-Block	unpublished		7.0	2.0	5.1	
Blood Meal	Kossel-Ayre	Ayre 38	12.6			6.7	
Blood Meal	Kossel-Ayre	Ayre 38	14.5			7.7	
Blood Meal	Kossel-Block	Ayre 38	13.7			6.2	
Tankage	Kossel-Block	unpublished	10.6	5.5	2.7	6.0	
"Stick Water"	Kossel-Block	unpublished	11.8	5.4	2.6	4.1	Fish

COMMENTS ON FEEDS

Feeds: With the exception of soybean protein, plant feeds are considerably lower in lysine than animal feeds.

Bread: The use of milk and yeast in the preparation of bread enhances its lysine content and consequently its nutritive value as compared to flour alone.

HORMONES, ENZYMES
Diamino Acids in
Hormones and Non-Metallic Enzymes
Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Insulin	Van Slyke	Jensen	318	16.0	3.3	4.5	2.3	
Insulin	Van Slyke	Jensen	320	(16.0)	3	8	2	
Insulin	Sakaguchi	Thomas	607	15.7	3.4*			
Insulin	Kossel-Block	Chibnall	160	15.5	3.2		1.3*	
Pepsin	Kossel-Calvery	Calvery	146	15.4	1.4	trace	1.8	
Pepsin	Kossel-Calvery	Calvery	146	15.2	1.2	trace	2.1	heat precipitate
Pepsin	Kossel-Calvery(?)	Calvery	146	. 15.4	1.8	trace	1.7	heat filtrate
Pepsin	Sakaguchi	Brand	127	(15.4)	1.0			swine
Thyro-globulin	Van Slyke	Cavett	152		7.4	1.8	5.8	normal human
Thyro-globulin	Van Slyke	Cavett	152		7.4	1.8	5.4	colloid goiter
Thyro-globulin	Van Slyke	Cavett	152		7.4	1.7	5.6	adenomatoses
Thyro-globulin	Van Slyke	Cavett	152		7.4	1.7	4.7	exophthalmic
Thyro-globulin	Van Slyke	Eckstein	202	15.6	8.2*	6.9	3.9	arg. by Kossel
Thyro-globulin	Kossel-Patten	Koch	373		5.8	3.1		'
Ribonuclease	Sakaguchi	Brand	127	(16.0)	5.2			
Yellow Enzyme	Sakaguchi, Knoop	Kuhn	393	16.3	8.1	2.8	13.6	lysine by N
Trypsin	Sakaguchi	Brand	127	(16.0)	3.3			
Trypsinogen	Sakaguchi	Brand	127	(16.0)	1.6			
Chymotryp-sinogen	Sakaguchi	Brand	127	(16.2)	2.8			
Lactogenic	Sakaguchi Thomas	Li	419	(16.0)	8.3			pituitary
Oxytocic	Sakaguchi-Dumazert	Potts	528	(16.0)	<0.8			pituitary
Pressor	Sakaguchi-Dumazert	Potts	528	(16.0)	12.3			pituitary
Gonado-tropin	Van Slyke	Evans	214		1.0	1.9	7.4	pregnant mare's serum
Secretin	Sakaguchi, Pauly	Ågren	25	14.4	7.3	4.4	9.9	Van Slyke lysine

* Best Values.

HORMONES AND ENZYMES

Pepsin: In line with its activity in strongly acid media, pepsin is deficient in the basic amino acids. The table shows again how the composition of a protein to be analyzed may be slightly changed

during the course of its preparation. Heat denaturation and coagulation of pepsin gave products which differed from the original.

Thyroglobulin: The values for the basic amino acids and especially for histidine given in the literature show such large discrepancies that it is difficult to surmise even the approximate composition of this protein.

Pituitary Hormones: The apparent lack of arginine in the oxytocic and the relatively large quantity in the pressor principle is but another example of the "unusual" composition of many of the physiologically active proteins and polypeptides as compared to the more "inert" tissue proteins.

KERATINS

Diamino Acids in *Eukeratins*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Human Hair	Kossel-Vickery	Vickery	647	(16.0)	8.0	0.5	2.5	
Human Hair	Kossel-Vickery	Vickery	656	16.9*	8.9*			direct
Human Hair	Kossel-Block	Block	97	15.4	8.3	1.0*	2.6*	
Wool	Kossel-Vickery	Vickery	648	16.6	8.3	0.6	2.2	
Wool	Kossel-Vickery	Vickery	656	16.6	10.0			direct
Wool	Kossel-Block	Block	97	15.4	9.4	0.7	2.8*	
Wool	Jansen-Graff	Graff	263		9.9			
Wool	Kossel-Block	Gordon	261		10.1*	0.7*	2.6	arg. direct
Wool	Kossel-Block	Sullivan	601	(16.0)	9.6	0.7	2.7	
Wool	Kossel-Block	Rutherford	561	(16.8)			3.1	
Wool	Van Slyke	Van Slyke	639		0.10 per cent HO·lysine			
Camel Hair	Kossel-Block	Block	97	15.1	9.6	1.0	3.2	
Chimpanzee Hair	Kossel-Block	Block	109	16.7	7.8	0.6	1.9	
Dog Hair	Van Slyke	Van Slyke	630		7.6	2.1	4.5	
Hog Hair	Kossel-Block	unpublished		15.1	8.7	1.1	2.5	
Goat Hair	Kossel-Block	Block	97	16.2	8.6	1.1	3.7	
Cow Hair	Kossel-Block	Block	109	15.5	7.7	0.7	2.1	
Cattle Horn	Kossel-Block	Block	80	15.1	10.4	0.7	3.2	
Cattle Horn	Kossel-Fürth	Fürth	251	(16.0)	(4.7)			
Cattle Horn	Kossel-Block	unpublished		14.8	7.4	1.1	2.6	
Rhinoceros Horn	Kossel-Block	Block	97	15.6	8.5	0.7	2.8	
Human Nails	Kossel-Block	Block	80	14.9	10.0*	0.5	2.8	
Human Nails	Kossel-Block	Hess	292	(14.9)	7.1	0.5*	2.8*	
Porcupine Quills	Kossel-Block	Block	91	15.8	8.1	0.6	2.6	
Echidna Spines	Kossel-Block	Block	91	15.2	7.4	0.6	1.9	
Goose Feathers	Kossel-Vickery	Block	97	15.5	5.2	0.4	1.1	
Hen Feathers	Kossel-Block	Block	97	15.5	6.5	0.7	1.8	
Eggshell	Kossel-Calvery	Calvery	141	(15.0)	7.8	0.6	2.2	
Eggshell	Kossel-Calvery	Calvery	142	16.6	8.6*	0.8*	3.5*	
Mean with 2×S.E.					8.4 ±0.2	0.79 ±0.15	2.6 ±0.3	

* "Best Values."

KERATINS

Diamino Acids in *Skin and Neurokeratins*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Human	Kossel-Vickery	Block	79	15.5*	6.2	0.9*	4.4*	skin
Human	Van Slyke	Eckstein	203	14.2	6.7	0.7	5.3	skin
Human	Kossel-Block	Wilkerson	678	15.1	10.6*	0.6	3.3	skin
Lamb	Kossel-Block	Sullivan	601	(16.0)	15.0	0.7	3.5	skin
Snake	Kossel-Vickery	Block	77	17.1	5.7	0.5	1.3	skin
Snake	Kossel-Block	Block	97	15.2	6.3	0.8	2.6	skin trypsin
Snake	Kossel-Block	unpublished		15.2	6.7*	1.0*	3.7*	skin undigested
Turtle	Kossel-Block	Block	96	14.1	6.4	1.7	2.0	scutes
Pelican	Kossel-Block	Block	96	14.0	6.9	1.4	4.0	excrescence
Whale	Kossel-Block	Block	96	14.1	7.0	1.6	4.3	baleen
Horse	Kossel-Block	Block	93	14.1	6.4	1.9	4.7	burrs
Tarpon	Kossel-Block	unpublished		16.3	9.4		4.6	scales
Neurokeratin	Kossel-Vickery	Block	78	14.1	3.9*	2.0	3.6	
Neurokeratin	Kossel-Kutscher	Argiris	32	14.2*	2.6	0.9	3.1	

* "Best Values."

KERATINS

Diamino Acids in *Egg Casings, Gorgonia, Silk Fibroin, etc.*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Scyllium	Kossel-Kutscher	Pregl	529	15.1	3.4	1.8	3.9	egg casing
Salmon	Kossel-Block	Young	694	15.3	6.1	1.4	3.7	egg casing
Herring	Kossel-Kutscher	Steudel	593	14.2	7.1	2.4	6.3	egg casing
Bombix Mori	Kossel-Block	unpublished		14.4	5.1	1.8	4.1	egg casing
Python	Kossel-Block	unpublished		14.1	5.8		1.4	egg casing
Gorgonia	Kossel-Vickery	Block	77	15.6*	7.5*	0.5	2.8	
Gorgonia	Kossel-Block	Block	96	14.1	5.9	1.4*	4.5*	
Plexaurella	Kossel-Vickery	Block	77	15.6*	6.9*	0.4	3.1	
Plexaurella	Kossel-Block	Block	96	13.8	6.5	1.8*	3.8*	
Sponge	Kossel-Block	Block	96	13.0	6.2*	present	4.3*	
Sponge	Van Slyke	Clancy	163		5.6	0.0	3.7	
Silk Fibroin	Kossel-Vickery	Vickery	650	19.0*	0.6	0.06	0.2	
Silk Fibroin	Kossel-Kutscher	Abderhalden	20	19.0	1.3	0.6	0.7	
Silk Fibroin	Kossel-Vickery	Bergmann	69	19.0	0.8			
Silk Fibroin	Kossel Fürth	Fürth	251	(19.0)	1.3			
Silk Fibroin	Kossel Vickery	Vickery	656	19.0	0.6*			direct

* "Best Values."

KERATINS

Eukeratins: The striking constancy of the molecular ratio of histidine to lysine to arginine as seen in the eukeratins (hair, horn, spines, feathers, etc.) definitely characterizes this group of proteins. As one would expect, the ratio of the three diamino acids is probably not absolutely constant in the large group of tissue proteins but the differences are small when compared to the obvious gross similarities.

LIVER PROTEINS

Diamino Acids in *Liver Proteins*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Human	Kossel-Block	Block	105	13.7	5.7	3.1	6.7	
Human	Kossel-Fürth	Fürth	251	(14.0)	8.5			
Cat	Kossel-Block	Block	87	13.9	6.7	2.2	5.9	corrected
Cat	Kossel, Knoop, Pauly	Urban	625	15.0	6.1	2.4	9.6	lysine by Van Slyke
Cat Globulin	Kossel, Knoop, Pauly	Urban	625	14.8	6.4	2.3	9.3	lysine by Van Slyke
Cat Albumin	Kossel, Knoop, Pauly	Urban	625	15.4	5.9	3.1	10.2	lysine by Van Slyke
Dog	Sakaguchi-Thomas	Thomas	607	(14.0)	6.7			
Dog	Kossel-Kutscher	Wakeman	670		4.6*	1.4*	4.0*	
Beef	Kossel-Block	unpublished		13.3	6.3	2.5	5.1	
Beef	Kossel-Block	Beach	59		6.6	2.0	6.0	
Sturgeon	Kossel-Kutscher	Wakeman	670		3.4*	1.1*	3.4*	
Cod	Kossel-Block	unpublished					5.0*	
"Best Values"				16.0	6.5	2.4	6.3	
					6.5±0.7	2.6±0.3		

* Omitted from Mean Values.

METALLOPROTEINS

Diamino Acids in *Metalloproteins* other than Hemoglobin

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Ferritin	Van Slyke	Kuhn	396	8.4	17.7*	0.5	4.8	
Hemocyanin	Van Slyke	Van Slyke	630		7.8	7.8	7.2	
Hemocyanin	Kossel-Block	Mazur	441	17.5	5.9*	4.2	8.2*	
Hemocyanin	Sakaguchi, Pauly	Roche	550	(15.6)	5.5	6.1*	8.0	Lysine by NH₂-N
Hemerythrine	Sakaguchi, Pauly	Roche	550	16.8	5.4	5.1	8.5	Lysine by NH₂-N
Cytochrome C	Sakaguchi, Pauly	Theorell	605	15.4	2.8	3.4	24.4	Lysine by N only
Cytochrome C	Kossel-Kutscher	Theorell	605	15.4			15.4*	

* Best Values.

METALLOPROTEINS

Ferritin is unusually rich in arginine and practically deficient in histidine. This is in contrast to hemoglobin, another Fe containing protein, which is very rich in histidine and but poorly supplied with arginine.

Hemocyanin is not as rich in histidine as is hemoglobin.

Cytochrome C is relatively deficient in arginine and histidine but unusually rich in lysine.

MILK PROTEINS

Basic Amino Acids in *Casein*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
			per cent	gm.	gm.	gm.	
Electrolytic-Kossel	Albanese	27	13.8	3.7	3.2	5.4	Lysine by N only
Kossel-Ayre	Ayre	38	14.5			6.8	
Kossel-Block	Beach	55	15.1	3.8	1.7	6.6	
Kossel-Block	Beach	57	14.5	4.2	2.0	6.8	
Kossel-Block	Block	85	16.1			7.2	
Van Slyke	Block	85	16.1			5.2	
Kossel-Calvery	Calvery	138		3.1	1.7	5.5	ave. of 10 detrs.
Van Slyke-Cavett	Cavett	151		3.8	2.7	8.7	
Van Slyke-Cavett	Cavett	153		4.0	2.9	9.6	
Knoop-Conrad	Conrad	168			2.6		
Kossel-Block	Csonka	181	16.0	3.8	2.5	7.6	Histidine by Knoop method
Kossel-Fürth	Fürth	251	(15.4)	5.4			
Jansen-Graff	Graff	263		4.1			
Pauly-Koessler	Hanke	278	(15.4)		3.0		
Pauly-Koessler	Hanke	280	(15.4)		2.7		
Kossel-Kutscher	Hart	283	15.9	4.9	2.6	5.8	
Jansen-Hunter	Hunter	313		4.0			
Knoop	Kapeller-Adler	351	15.4		4.3		
Kossel-Gross	Kossel	384		4.5			
Sakaguchi & Knoop	Kuhn	393	(15.4)	3.8	1.4	9.5	Lysine by N
Harden-Lang	Lang	399	15.4	3.4			
Lautenschläger	Lautenschläger	405	(15.4)		3.7		HI hydrolysis on 1,825 gm.
Lieben	Lieben	420	13.6			6.8[a]	
Kossel-Kutscher	Leavenworth	410	15.6			5.9	
Van Slyke	Narayana	471		4.7	2.4	6.7	
Orglmeister	Orglmeister	487	(15.4)	3.7			
Kossel-Patten	Osborne	494	15.6	3.9	2.6	6.1	
Van Slyke	Plimmer	521	15.2	3.9	1.6	9.9	
Kossel-Block	Plimmer	521	15.2	3.9	1.8	6.4	
Kossel-Block	Plimmer	523	14.1	3.6	1.7	5.1	
Kossel-Block	Pottinger	527	(15.4)	5.4	2.7	7.9	
Sakaguchi	Sakaguchi	563		4.2			
Sakaguchi-Thomas	Thomas	607	(15.4)	3.5–4.3			
Kossel-Block	Tristram	619		4.2	1.9	6.6	
Van Slyke	Van Slyke	639					0.31 per cent HO·lysine
Kossel-Osborne	Van Slyke	631	-	4.0[a]	2.7[a]	7.9[a]	
Kossel-Vickery	Vickery	654	15.5	4.1	1.9	6.5	
Kossel-Vickery	Vickery	656	15.5	3.8			direct precipitation
"Best Values"			15.5	4.2	2.5	7.5	
Mean with 2×S.E.				4.1±0.2	2.5±0.3	6.9±0.7	
Kossel-Block	Beach	57	13.7	6.0*	1.3	6.8*	Human Casein
Kossel-Block	Plimmer	521	14.4	4.0	1.7*	6.1	Human Casein
Van Slyke	Plimmer	521	14.4	3.6	1.2	7.0	Human Casein

* "Best Values."

[a] Omitted from mean.

MILK PROTEINS

Basic Amino Acids in *Lactalbumin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE	
			per cent	gm.	gm.	gm.	
Kossel-Block	Beach	57	14.2	3.9	1.6	8.9	cow's milk
Kossel-Block	unpublished		13.8	3.7	2.0	.9.6	cow's milk
Kossel-Kutscher	Osborne	505	15.5	3.1	1.6	9.1	cow's milk
Van Slyke	Osborne	505	15.5	3.6	2.7	10.2	cow's milk
Van Slyke	Plimmer	521	14.2	4.5	2.3	9.1	cow's milk
Kossel-Block	Plimmer	521	14.2	4.5	2.0	7.1	cow's milk
Van Slyke	Van Slyke	639		0.03 per cent hydroxylysine			cow's milk
Jansen-Graff	Graff	263		2.8			cow's milk
"Best Values"			15.5	3.9	2.0	9.6	
Kossel-Block	Beach	57	13.7	6.0*	1.3	6.8	human milk
Kossel-Block	Plimmer	521	14.6*	5.5	1.9*	7.2*	human milk
Kossel-Block	Plimmer	521	14.6	5.1	0.8	10.7	human milk

* "Best Values."

MILK PROTEINS

Basic Amino Acids in *β-Lactoglobulin, Whole Milk, and Casein Hydrolysates*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE	
				per cent	gm.	gm.	gm.	
β-Lactoglobulin	Kossel-Block	Bolling	112	15.5*	3.5*	1.8*	10.2	
β-Lactoglobulin	Sakaguchi-Weber	Brand	128	15.6	2.9			
β-Lactoglobulin	Kossel-Block	Cannan	147	15.5	2.9	2.4	10.9*	
β-Lactoglobulin	Van Slyke	Van Slyke	639	16.0	0.02 per cent hydroxylysine			
β-Lactoglobulin	Kossel-Chibnall	Chibnall	160	15.6	3.0	1.6	10.0	
Casein—	Kossel-Block	unpublished		12.2	6.0	3.2	8.0	Borden
Hydrolysates	Kossel-Block	unpublished		12.3	6.0	3.6	8.0	Borden
Hydrolysates	Kossel-Block	unpublished		7.4	4.1	2.8	7.7	Difco
Hydrolysates	Kossel-Block	unpublished		10.5	4.4	3.2	9.1	Difco
Whole Milk	Kossel-Block	unpublished		15.2	4.3	2.5	7.5	Cow
Whole Milk	Kossel-Block	unpublished		15.2	5.0	2.7	7.2	Human

* "Best Values."

MILK PROTEINS

Casein: This protein, although probably not homogeneous, has been analyzed more often than any other protein. The composition of human and cow caseins appears to differ.

Lactalbumin or whey protein (cow) is richer in lysine than is casein but it appears to yield less histidine.

β-Lactoglobulin which is the most homogeneous protein isolated to date from milk, has recently been very carefully analyzed for the

diamino acids. It is considerably richer in lysine, but is poorer in histidine than casein.

Whole human milk proteins yield significantly more arginine than do cows' milk proteins.

MUSCLE PROTEINS

Basic Amino Acids in *Animal Muscle Proteins*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE
				per cent	gm.	gm.	gm.
Beef Muscle	Kossel-Block	Beach	59		6.9	2.3	8.1
Beef Muscle	Kossel-Block	unpublished		16.1	7.1	2.2	8.0
Beef Muscle	Kossel-Fürth	Fürth	251	(16.0)	5.7		
Beef Muscle	Kossel-Patten	Osborne	498	16.2	7.4	1.7	7.5
Beef Muscle	Kossel-Block	Pottinger	527	(16.0)	7.5	1.8	7.6
Beef Muscle	Kossel-Vickery	Rees	542	15.9	6.3	0.6	7.3
Veal Muscle	Kossel-Block	Beach	59		7.5	2.4	9.6
Lamb Muscle	Kossel-Block	Beach	59		7.6	2.4	8.7
Pork Muscle	Kossel-Block	Beach	59		6.6	2.2	8.7
Rat—Normal	Van Slyke-Pauly	Roche	548	15.8	9.5	3.4	9.9
Rat—Protein Starved	Van Slyke-Pauly	Roche	548	16.3	8.9	3.3	5.6
Rabbit Myosin	Kossel-Block	Sharp	575	16.8	6.7	1.6	9.4
Rabbit Myogen	Kossel-Block	Sharp	575	(16.0)	6.0	2.8	7.7
Rabbit Muscle		Kandatu	349	(16.0)	6.9	1.1	9.6
Chicken—light	Kossel-Block	Beach	59		6.9	2.3	8.4
Chicken—dark	Kossel-Block	Beach	59		7.1	2.3	8.4
Turtle	Kossel-Block	Beach	59		6.7	2.3	7.7
Mean with 2×S.E.					7.1±0.5	2.2±0.4	8.1±0.6

MUSCLE PROTEINS

Basic Amino Acids in *Fish Muscle Proteins*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE	
			per cent	gm.	gm.	gm.	
Kossel-Block	Abderhalden	24	13.6	8.0	5.6	9.4	Cod
Kossel-Block	Pottinger	527	(16.0)	5.6	1.7	6.8	Cod
Kossel-Block	Pottinger	527	(16.0)	5.8	1.4	6.8	Croaker
Kossel-Patten	Osborne	493	(16.0)	6.3	2.6	7.5	Halibut
Kossel-Block	Pottinger	527	(16.0)	6.0	1.7	6.2	Halibut
Kossel-Block	Pottinger	527	(16.0)	5.7	1.2	6.4	Haddock
Kossel-Block	Pottinger	527	(16.0)	5.1	1.6	7.0	Herring
Kossel-Block	Pottinger	527	(16.0)	5.7	1.4	7.2	Lake Trout
Kossel-Block	Pottinger	527	(16.0)	5.8	1.9	7.1	Mackerel
Kossel-Block	unpublished		11.6	5.9	2.4	5.7	Menhaden meal
Kossel-Block	Pottinger	527	(16.0)	5.8	1.6	6.7	Mullet
Kossel-Block	Pottinger	527	(16.0)	5.6	1.2	6.8	Pilchard
Kossel-Block	Pottinger	527·	(16.0)	6.2	1.6	6.7	Red Snapper
Kossel-Block	Pottinger	527	(16.0)	5.4	1.5	6.2	Salmon
Kossel-Block	Beach	59		6.4	2.3	9.0	Salmon
Kossel-Block	Pottinger	527	(16.0)	4.5	1.1	6.5	Shad
Kossel-Block	Pottinger	527	(16.0)	5.9	1.4	6.8	Sea Trout
"Best Values"			16.0	6.0	2.0	8.0	
Mean with 2×S.E.				5.6±1.0	1.9±0.6	6.7±0.4	

MUSCLE PROTEINS
Basic Amino Acids in *Crustacean Proteins*

Calculated to 16.0 gm. N.

SPECIES	METHOD	REFERENCE	NITROGEN	ARGININE	HISTIDINE	LYSINE
			per cent	gm.	gm.	gm.
Clam	Kossel-Block	Pottinger 527	(16.0)	5.3	1.5	5.4
Crab	Kossel-Block	Pottinger 527	(16.0)	7.6	1.5	6.4
Oyster	Kossel-Block	Pottinger 527	(16.0)	5.7	1.8	5.2
Scallop	Kossel-Patten	Osborne 496	17.1	6.9	1.9	5.4
Shrimp	Kossel-Block	Pottinger 527	(16.0)	5.7	1.8	5.2
Shrimp	Kossel-Block	Beach 59		6.6	1.8	8.3
"Best Values"			16.0	6.5	1.8	8.0
Mean with 2×S.E.				6.2±0.9	1.7±0.2	5.5±0.5

MUSCLE PROTEINS

The relative constancy in the basic amino acids in all types of muscle, animal, fish, or crustacean, is noteworthy.

PLANT PROTEINS
Diamino Acids in *Autotropic Organisms (Algae, Fern, etc.)*

Calculated to 16.0 gm. N.

ORGANISM	METHOD	REFERENCE	ARGININE	HISTIDINE	LYSINE	
			gm.	gm.	gm.	
Phormidium	Kossel-Block	Mazur 442	4.6	3.8	0.0	Alga
Ulva	Kossel-Block	Mazur 442	3.7	0.7	0.0	Alga
Laminaria	Kossel-Block	Mazur 442	8.0	0.9	0.0	Alga
Sargassum	Kossel-Block	Mazur 442	4.0	1.9	4.5	Alga
Gloeotrichia	Kossel	Mazur 443	1.3	0.9	1.7	Alga
Macrocystis	Kossel	Mazur 443	4.4	0.9	1.6	Alga
Lessoniopsis	Kossel	Mazur 443	1.6	0.7	6.5	Alga
*Fucus	Kossel	Mazur 443	0.0	0.6	6.1	Alga
*Cystoseira	Kossel	Mazur 443	0.0	2.5	3.5	Alga
*Egregia	Kossel	Mazur 443	0.0	2.4	0.3	Alga
Caulerpa	Kossel	Mazur 443	3.0	1.8	0.0	Alga
Codium	Kossel	Mazur 443	3.6	2.7	4.5	Alga
Chondrus	Kossel-Block	Mazur 442	5.1	1.1	3.4	Irish Moss
Osmunda	Kossel-Block	Mazur 442	3.8	2.2	3.0	Fern
Diatoms	Kossel	Mazur 443	0.9	5.0	3.7	
Pteridium	Kossel-Block	Lugg 433A	7.6†	1.4†	5.4†	Fern

* The only known tissue proteins which do not contain arginine (sic).
† Best Values.

PLANT PROTEINS

Diamino Acids in Corn (Zea Mays) Kernel Proteins other than Zein

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Corn	Kossel-Block	unpublished			4.8	2.5	2.9	yellow
Corn	Kossel-Block	Csonka	181	1.78	2.0	1.0	1.0	yellow
Corn	Kossel-Block	unpublished			3.1	2.2	2.1	white
Corn	Kossel-Block	Csonka	181	1.71	1.9	0.8	1.0	white
Gluten	Kossel-Block	unpublished		12.7	3.1	1.6	0.8	yellow
Gluten meal	Kossel-Block	unpublished		13.4	3.2	1.9	2.0	yellow
Gluten	Kossel-Block	unpublished		10.9	3.0	1.6	1.5	white
Germ	Kossel-Block	unpublished		12.8	8.1	2.9	5.8	yellow
Germ	Kossel-Block	unpublished		11.8	5.5	2.4	4.2	white
Zein residue	Kossel-Block	unpublished		10.9	2.9	1.6	1.6	yellow
Bran	Kossel-Block	unpublished				3.4		yellow
Albumins	Kossel-Block	unpublished		13.8	5.4	6.7	1 to 2	"steep water"
Albumins	Kossel-Patten	Osborne	490	(16.0)	7.1	3.0	2.9	NaOH soluble
Glutelin	Kossel-Patten	Osborne	494	(17.0)	6.7	2.8	2.8	

PLANT PROTEINS

Diamino Acids in Edestin

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE	
			per cent	gm.	gm.	gm.	
Electrolytic-Kossel	Albanese	27	17.3	13.1	2.5	1.7	lysine by N only
Kossel-Block	Beach	55	17.1	13.6	1.4	1.7	
Kossel-Calvery	Calvery	138		12.8	3.1	3.0	
Kossel-Block	Gordon	261		14.0	2.0	2.0	arginine directly
Pauly-Koessler	Hanke	278	(17.1)		2.8		
Jansen-Hunter	Hunter	313		13.3			
Kossel-Pauly	Kiesel	363	18.4	13.3	3.5	3.5	
Kossel-Pauly	Kiesel	363	18.4	13.3	4.5	3.6	heat coagulated
Kossel-Patten	Kossel	380	(18.4)	12.5	1.8	1.5	
Kossel-Gross	Kossel	384		12.4			
Kossel-Patten	Osborne	494	18.4	12.5	2.1	1.5	
Sakaguchi	Sakaguchi	563		13.4			
Kossel-Vickery	Vickery	656	18.7	14.3			direct
Sakaguchi	Thomas	607	18.4	16.1			
Kossel-Block	Tristram	619		13.4	1.8	2.2	
Van Slyke	Van Slyke	630		13.5	3.4	3.3	
Kossel-Vickery	Vickery	643	18.4	13.8	1.8	1.9	
Kossel-Vickery	Vickery	659	(18.4)		2.3		
Kossel-Chibnall	Chibnall	160	18.7	14.3	2.1	2.0	
"Best Values"			18.7	14.3	2.3	2.0	
Mean with 2 ×S.E.				13.5 ± 0.4	2.3 ± 0.5	2.4 ± 0.5	

PLANT PROTEINS
Diamino Acids in *Gliadin*
Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE	
			per cent	gm.	gm.	gm.	
Kossel-Kutscher	Abderhalden	7	(17.7)	3.1	1.5	0.0	
Van Slyke	Cavett	153		2.3	0.9	1.3	
Jansen-Graff	Graff	263		2.7			
Pauly-Koessler	Hanke	280	(17.7)		1.9		
Jansen-Hunter	Hunter	313		2.4			
Kossel-Kutscher	Kossel	379	(17.7)	2.5	1.1	0.0	
Kossel-Patten	Osborne	489	(17.7)	2.9	0.5	0.0	
Lieben-Loo	Lieben	420	(17.7)			1.2	
Sakaguchi	Sakaguchi	563		2.5			
Kossel-Kutscher	Osborne	505	17.5	2.5	1.4	0.6	
Van Slyke	Osborne	505	17.5	2.7	2.0	1.1	
Van Slyke	Van Slyke	630		2.8	3.1	?	
Van Slyke	Van Slyke	639		0.11 per cent hydroxylysine			
Kossel-Vickery	Vickery	656	17.7	2.3			direct method
"Best Values"			17.7	2.7	1.9	?	
Mean with 2 ×S.E.	.			2.6±0.2	1.6±0.5	0.5	

PLANT PROTEINS
Diamino Acids in *Grasses*
Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		ARGININE	HISTIDINE	LYSINE
				gm.	gm.	gm.
Cocksfoot	Kossel-Vickery	Miller	451	6.4	0.9	4.1
Cocksfoot	Kossel-Block	Miller	451	6.1	1.3	
Cocksfoot	Van Slyke	Miller	451	6.8	4.2	6.8
Cocksfoot	Kossel-Block	Tristram	619	7.7	1.5	5.3
Perennial Rye	Kossel-Block	Miller	451	6.3	1.6	4.5
Perennial Rye	Kossel-Block	Tristram	619	7.2	1.4	5.2
Italian Rye	Kossel-Block	Tristram	619	6.7	1.4	5.0
Meadow	Kossel-Block	Miller	451	6.5	1.1	4.5
Meadow	Kossel-Block	Tristram	619	8.2	1.4	4.7
Fescue	Kossel-Block	Miller	451	6.0	1.4	4.5
Fescue	Kossel-Block	Tristram	619	7.3	1.2	4.8
Timothy	Kossel-Block	Miller	451	6.1	1.4	3.8
Autumn	Kossel-Block	Miller	451	6.5	1.1	4.5
Dogstail	Kossel-Block	Tristram	619	7.5	1.5	4.9
Lucerne	Kossel-Block	Tristram	619	7.5	1.4	5.9
"Best Values"				7.3	1.6	5.0
Mean with 2 ×S.E.				6.9±0.4	1.5±0.4	4.9±0.5

PLANT PROTEINS
Diamino Acids in *Leaf Proteins*
Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITROGEN	ARGININE	HISTIDINE	LYSINE
				per cent	gm.	gm.	gm.
Alfalfa	Kossel-Block	unpublished		10.6	4.3	2.1	4.2
Clover, red	Kossel-Block	Miller	451		6.2	1.4	3.7
Clover, red	Kossel-Block	Tristram	619		7.4	1.4	5.5
Clover, white	Kossel-Block	Tristram	619		7.7	0.9	5.6
Spinach	Kossel-Block	Tristram	619		7.0	1.3	5.2
Beet tops	Kossel-Block	Tristram	619		6.5	1.4	5.7
Corn leaves	Kossel-Block	Tristram	619		7.2	1.2	5.1
"Best Values"					7.0	2.1	5.4
Mean with 2 ×S.E.					6.6±1.0	1.4±0.3	5.0±0.4

PLANT PROTEINS

Diamino Acids in *Miscellaneous Plant Proteins*

Calculated to 16.0 gm. N.

PLANT	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Cottonseed	Kossel-Patten	Osborne	494	18.6*	11.6	3.0	2.0	globulin
Cottonseed	Van Slyke	Cavett	153		12.2	2.3	5.0	globulin
Cottonseed	Kossel-Block	Fontaine	238	17.9	12.2	3.0*	5.2*	globulin
Cottonseed	Van Slyke	Van Slyke	639		0.21 per cent HO·lysine			globulin
Cottonseed	Kossel-Vickery	Vickery	656	18.6	12.8*			globulin
Cottonseed	Kossel-Block	unpublished		10.9	7.4	2.6	2.7	meal
Castor bean	Kossel-Block	Tristram	619		6.4	1.3	5.5	
Linseed	Kossel-Block	unpublished			6.2	1.5	2.5	meal
Peanut	Van Slyke-Cavett	Brown	134	18.0	11.1	1.9	4.0	arachin
Peanut	Van Slyke-Cavett	Brown	134	18.0	12.7	1.9	6.0	conarachin
Peanut	Kossel-Ayre	Ayre	38	7.6			2.8	cake
Peanut	Kossel-Block	unpublished		10.4	9.9	2.1	3.0	meal
Peanut	Kossel-Gross	Kossel	384		15.4			arachin
Peanut	Kossel-Kutscher	Johns	322	18.3*	11.0	1.9*	1.5	arachin
Peanut	Van Slyke	Johns	322	18.3	11.9	1.6	4.4	arachin
Peanut	Kossel-Vickery	Vickery	656	18.3	12.2*·			arachin
Cucurbit seed	Kossel-Vickery	Vickery	657	18.5	13.8 to 15.4			4 species
Soybean	Kossel-Block	unpublished			5.8	2.3	5.4	meal
Soybean	Kossel-Pauly	Kiesel	362	17.5	8.5	3.3		glycinin
Adsuki bean	Kossel-Kutscher	Jones	341	16.6	5.1	1.7	4.0	globulin
Pea	Kossel-Patten	Osborne	491	17.1	8.3	2.1	5.0	vicilin
Pea	Kossel-Patten	Osborne	492	18.0	4.9	2.0	2.7	legumelin
Rubber	Kossel-Block	Tristram	620	15.0	6.5	0.6	5.3	
Hordein	Kossel-Patten	Osborne	494	17.2*	2.0	1.2	0.0	
Hordein	Kossel-Kutscher	Kleinschmidt	369	17.2	2.9*	0.5	0.0	
Ricin	Kossel	Karrer	355	17.0	11.7	0.0	6.3	Lysine from N

* "Best Values."

PLANT PROTEINS

Diamino Acids in *Oat and Rice Proteins*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Oats	Kossel-Block	unpublished			5.8	2.0	3.3	Cereal
Oats—whole	Kossel-Block	Csonka	182		2.1	0.6	1.8	Richland
Oats—whole	Kossel-Block	Csonka	182		3.7	0.9	1.3	Commercial
Oats—rolled	Kossel-Block	Csonka	182		3.9	1.3	1.1	
Oats—middlings	Kossel-Block	Csonka	182		3.8	1.2	0.6	
Oats—shorts	Kossel-Block	Csonka	182		3.5	0.7	1.3	
Rice	Kossel-Block	Kik	364		3.3	0.8	3.4	Whole
Rice	Kossel-Block	Kik	364		3.9	0.9	4.4	Polished
Rice	Kossel-Block	Kik	364		2.6	0.7	3.3	Bran
Rice	Kossel-Block	Kik	364		2.2	0.6	3.0	Polish
Rice	Kossel-Block	Kik	364		3.4	0.6	3.6	Arkansas 155
Rice	Kossel-Block	Kik	364		3.7	1.1	4.2	Shoemed
Rice	Kossel-Block	Kik	364		4.2	0.6	4.1	Acadia
Rice	Kossel-Block*	Kik	364		3.9	0.9	3.9	Zenith
Rice	Kossel-Block	Kik	364		3.5	0.9	5.1	Fortuna
Rice	Van Slyke	Osborne	505	16.7	8.8	3.2	4.1	Oryzenin
Rice	Kossel-Block	unpublished			7.2	1.5	3.2	Cereal

PLANT PROTEINS

Diamino Acids in *Wheat Proteins* other than Gliadin

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Wheat	Kossel-Block	unpublished			3.8*	2.1*	2.7*	Soft Spring
Wheat	Kossel-Block	Csonka	179		2.5	1.4	(7.3)	Hard Marquis
Wheat	Kossel-Block	Csonka	179		2.3	0.7	(7.6)	Hard Tenmarq
Wheat	Kossel-Block	Csonka	179		2.4	0.5	(6.0)	Soft Fulhio
Gluten	Kossel-Block	unpublished		13.5	3.9*	2.2*	1.9*	5 samples
Gluten	Kossel-Ayre	Ayre	38	12.6			1.3	
Gluten	Kossel-Ayre	Padoa	508		1.9	1.0	1.2	
Flour	Kossel-Block	Csonka	180	3.02	2.2	1.0	(5.2)	Hard Marquis
Flour	Kossel-Block	Csonka	180	2.13	2.5	0.8	(6.5)	Hard Tenmarq
Glutenin	Kossel-Patten	Osborne	489	17.5	4.3	1.6	1.7	
Glutelin	Van Slyke	Csonka	175	17.1	5.8	3.5	2.8	
Germ	Kossel-Block	unpublished			6.0*	2.5*	5.5*	
Germ	Kossel-Patten	Osborne	489	16.8	5.6	2.7	2.7	Leucosine
Bran	Kossel-Block	Csonka	180	2.75	1.9	0.3	(10.8)	Tenmarq
Shorts	Kossel-Block	Csonka	180	3.22	3.4	0.3	(9.9)	Tenmarq

PLANT PROTEINS

Biologically Active Substances
Diamino Acids in *Viruses and Allergens*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Virus	Sakaguchi, Knoop	Ross	556	16.0	9.0	0.0		Tobacco mosaic
Virus	Kossel-Vickery	Ross	557	15.9*	10.1*	0.0	0.0	" "
Virus	Kossel-Pauly	Knight	371	(16.0)	9.2	0.0		Tobacco mosaic
Virus	Kossel-Pauly	Knight	371	(16.0)	10.0	0.0		Yellow aucuba
Virus	Kossel-Pauly	Knight	371	(16.0)	10.0	0.0		Green aucuba
Virus	Kossel-Block	Knight	371	(16.0)	9.2	0.6		Holmes' ribgrass
Virus	Kossel-Pauly	Knight	371	(16.0)	9.0	0.0		Holmes' masked
Virus	Kossel-Pauly	Knight	371	(16.0)	9.2	0.0		J 14D1
Virus	Kossel-Pauly	Knight	371	(16.0)	8.7	0.0		Cucumber 1
Virus	Kossel-Pauly	Knight	371	(16.0)	8.8	0.0		Cucumber 3
Allergen	Kossel-Vickery	Spies	585	19.8	27.3	trace	3.1	Cottonseed
Allergen	Kossel-Vickery	Spies	585	20.2	26.3	trace	1.3	Cottonseed
Allergen	Kossel-Vickery	Spies	585	11.6	40.7	0.0	2.8	Cottonseed
Lipoprotein		Balls	48	17.4	21.5			

* "Best Values"

PLANT PROTEINS

Diamino Acids in Yeast and Mold Proteins

Calculated to 16.0 gm. N.

	METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
				per cent	gm.	gm.	gm.	
Yeast	Kossel-Block	Block	87	10.0	4.7		6.3	high protein
Yeast	Kossel-Block	Block	87	9.0	4.6		6.1	low protein
Yeast	Kossel-Block	Block	87	8.3	4.7		6.3	starch-free
Yeast	Kossel-Block	Block	87	8.4	5.4		6.3	$(NH_4)_2SO_4$**
Yeast	Kossel-Block	Block	87	8.5	5.3		6.1	asparagine**
Yeast	Kossel-Block	unpublished		14.4	4.3	3.0	6.4	brewer's
Yeast	Kossel-Vickery	Csonka	178	(8.0)	2.6		4.3	brewer's
Yeast	Kossel-Block	unpublished			4.8	2.8	6.5	brewer's
Yeast	Kossel-Block	unpublished			4.5	2.9	6.9	brewer's
Yeast	Kossel-Block	Csonka	178	(8.0)	2.7		5.2	baker's
Yeast	Kossel-Block	unpublished			4.1	2.6	6.4	baker's
Yeast	Kossel-Knoop	Woolley	691			2.1		baker's
Yeast	Kossel-Block	unpublished			4.3	3.4	5.1	"steep-water"
Mold*	various	Woolley	689	5.15	1.8	0.5	2.5	aspergillus
Mean with 2×S.E.					4.3±0.5	2.8±0.3	6.0±0.4	

* Not included in Mean
** Medium

PLANT PROTEINS

Diamino Acids in Zein

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
			per cent	gm.	gm.	gm.	
Brazier	Brazier	129	17.5	(0.5)	1.3	0.0	
Pauly-Koessler	Hanke	280	15.5		1.3		
Lieben-Loo	Lieben	420				0.0	
Kossel-Kutscher	Kossel	379	(16.0)	1.8	0.8	0.0	
Kossel-Patten	Osborne	490	16.1	1.2	0.4	0.0	
Kossel-Patten	Osborne	494	16.1	1.2	0.8	0.0	
Sakaguchi	Sakaguchi	563		2.0			
Van Slyke	Van Slyke	639		0.31 per cent hydroxylysine			
Kossel-Vickery	Vickery	656	16.1	1.6			direct method
Kossel-Vickery	Vickery	655	(16.0)	1.6	0.8	0.0	
Kossel-Kutscher (?)	Leavenworth	655	(16.0)	1.5	0.8	0.0	
"Best Values"			16.1	1.6	0.9	0.0	
Mean with 2×S.E.				1.6±0.2	0.9±0.2	0.0	

PLANT PROTEINS

Plant proteins may vary in composition with species, climate, fertilization, etc.

Autotropic Organisms: These proteins were extracted from the dried organisms with hot 90 per cent formic acid. The analytical results, if true, indicate that here for the first time we have proteins

which are completely devoid of arginine. The reported wide variations in the quantities of lysine from 0.0 to 6.5 per cent, is also contrary to the usual experience with tissue proteins of *entire* plants and animals (*cf.* Lugg, 433A).

Corn: With the exception of the germ proteins, corn proteins are deficient although not lacking in lysine. Corn albumins, "steep water proteins," are one of the richest sources of histidine.

Edestin: Although probably not homogeneous this protein has been repeatedly analyzed. It, in common with many other seed globulins, is very rich in arginine.

Gliadin: This alcohol soluble protein from wheat gluten is poor in arginine and histidine and is deficient or devoid of lysine. The lysine values should be repeated.

Grasses: These proteins are relatively rich in arginine and contain about the same quantities of lysine that are present in corn and wheat germs and in soybeans. It is the authors' opinion that the values for histidine will be found to be higher than those given in the table when the nitranilic acid method is used in place of the diflavianate procedure.

Leaf Proteins: These are similar in composition to the grass proteins and are much superior in lysine to the commonly used seed proteins, i.e., wheat and corn.

Cottonseed Globulin: This protein contains ample quantities of lysine and histidine and is very rich in arginine but unfortunately it comprises only approximately 30 per cent of the total proteins of the cottonseed.

Soybean compares in composition with leaf, grass, and germ proteins and is but slightly, though significantly, inferior to animal proteins in lysine.

Oats and Rice: Our preliminary studies indicate that oat proteins are superior in diamino acids, especially lysine, to rice, corn and wheat. These results are not in accord with Csonka (182) and Kik (364).

Wheat: Csonka (179) has shown that there are species differences in the amino acid composition of whole wheat. Wheat proteins, like corn, are low in lysine except the germ proteins which contain ample quantities. The values (179) of 6.0 to 7.6 per cent of lysine in whole wheat are probably erroneous.

Viruses and Allergens: The relatively high content of arginine and the lack of histidine characterizes the chemical composition of these biologically active proteins and polypeptides.

Yeasts: Yeast proteins furnish a relatively large quantity of lysine and consequently are of use in improving the nutritive value of

bread and similar products. The sample of yeast which was grown on "steep water," corn albumins, appears to be richer in histidine and poorer in lysine than the other yeasts. This may be due to the inclusion of some of the histidine rich, lysine poor corn albumins in the preparation analyzed. The experiment shows the ability of microorganisms to convert a biologically poor protein into one of high nutritive value.

Zein: Zein, like gliadin, is deficient in arginine and histidine. It is devoid of lysine.

TISSUE PROTEINS

Basic Amino Acids in *Miscellaneous Tissue and Organ Proteins*

Calculated to 16.0 gm. N.

ORGAN	METHOD	REFERENCE	NITRO-GEN	ARGI-NINE	HISTI-DINE	LYSINE	
			per cent	gm.	gm.	gm.	
Kidney	Kossel-Block	unpublished	15.6	6.3	2.0	5.2	Beef
Kidney	Kossel-Block	Block 105	14.2	6.3	2.7	5.5	Human
Lung	Kossel-Block	unpublished	15.3	6.5	2.5	5.4	Beef
Lung	Kossel-Block	Beach 59		6.3	1.9	5.8	Beef
Lung	Kossel-Fürth	Fürth 251	(16.0)	6.5			Beef
Pancreas	Kossel-Block	unpublished	15.5	6.1	2.7	5.1	Beef
Salivary Gland	Kossel-Block	unpublished	15.7	6.5	2.7	5.8	Beef
Spleen	Kossel-Block	unpublished	15.7	8.3	2.1	6.1	Beef
Thymus	Kossel-Block	unpublished	15.4	7.2	2.4	6.1	Beef
Ovaries	Kossel-Block	unpublished	15.8	8.4	1.6	5.0	Beef
Testes	Kossel-Block	unpublished	15.4	8.6	2.1	6.1	Beef
Heart	Kossel-Block	unpublished	14.8	6.4	2.7	7.4	Beef
Heart	Kossel-Block	Beach 59		7.4	2.1	7.1	Beef
Heart	Kossel-Fürth	Fürth 251	(16.0)	6.5			Beef
Bladder	Kossel-Block	unpublished	15.9	6.5	1.5	5.0	Beef
Intestine	Kossel-Block	unpublished	15.3	7.8	1.9	6.0	Beef
Stomach	Kossel-Block	Beach 59		6.6	1.7	5.8	Beef
Protamine	Kossel-Gross	Kossel 384		44.2			Salmon
Protamine	Kossel & Pauly	Lizzitzin 421		39.1	6.2	5.5	Sturgeon

THE AROMATIC AMINO ACIDS

TYROSINE, TRYPTOPHANE, PHENYLALANINE, DIIODOTYROSINE, AND THYROXINE

	Tyrosine	Trypto-phane	Phenyl-alanine	Diiodo-tyrosine	Thyroxine
Empirical Formula	$C_9H_{11}O_3N$	$C_{11}H_{12}O_2N_2$	$C_9H_{11}O_2N$	$C_9H_9O_3NI_2$	$C_{16}H_{11}O_4NI_4$
Optical form	l	l	l	l	l
Molecular Weight	181.09	204.11	165.09	432.91	776.82
Carbon	59.64	64.67	65.41	24.97	23.17
Hydrogen	6.12	5.93	6.72	2.10	1.43
Nitrogen	7.74	13.72	8.49	3.24	1.80
Oxygen	26.50	15.68	19.38	11.09	8.24
Iodine				58.63	65.35
Melting Point	314–8° (cor.)	289° (cor.)	283° (cor.)	202° (uncor.)	235–6° (cor.)

PART I

HYDROLYSIS

INTRODUCTION: Hydrolytic losses have been briefly discussed in Chapter I (*cf.* references to the papers of Kossel and Kutscher (379), Hunter and Dauphinée (313), Tristram (619), Roche and Blanc-Jean (551), Block and Bolling (105), and others. While the literature pertaining to the destruction of the basic amino acids during hydrolysis is relatively small, that on tyrosine and especially on tryptophane is quite extensive. This is the result of two facts, the quantities of the aromatic amino acids are more easily determinable and secondly, instability of tryptophane, especially to acid hydrolysis, was recognized even before its isolation in 1901 by Hopkins and Cole (307).

The earlier literature on this subject has been adequately reviewed by Mitchell and Hamilton (462). Some more recent papers pertaining to destruction during protein hydrolysis will be mentioned here.

Tyrosine: Jorpes (344), in a careful study of the Millon-Folin (233) method, found that 91 to 102 per cent or a mean of 95.8 ± 0.9 per cent (17 determinations) of free tyrosine could be recovered after heating at 100° for 14 to 18 hours with 5 N NaOH.

Lugg (430) found that pure tyrosine is completely unaffected by

heating for 20 to 30 hours at 100° with 7 N H_2SO_4, 5 N NaOH, or 5.5 N NaOH containing 5 per cent $SnCl_2$. However, he showed that heating with 7 N H_2SO_4 in the presence of carbohydrate resulted in a considerable destruction of the amino acid.

Bolling and Block (112), as a result of 38 experiments on the recovery of tyrosine added to lactoglobulin (a protein which appears to be devoid of carbohydrate), and 6 experiments with tyrosine alone, report a loss of from 11 to 17 per cent of the added tyrosine after 5 hour hydrolysis with 5 N NaOH in an oil bath at 115 to 125°. They suggest a correction of 1.18, based upon an average recovery of 85 per cent, to be applied with this protein and under these conditions of hydrolysis.

Tryptophane: The experiments of Homer (305) and of Gortner and his associates (*cf.* 462) showed that tryptophane is destroyed by hot mineral acids in the presence of $CuSO_4$, $Fe_2(SO_4)_3$, carbohydrates, aromatic aldehydes, and aliphatic aldehydes. However, in the absence of these mineral salts and aldehydes, tryptophane is very resistant to destruction by mineral acids. The findings of Homer, in 1915, on the stability of tryptophane to hot sulfuric acid (305) have been recently confirmed by Hotchkiss (310) who reported that only 0.7 per cent of pure tryptophane is destroyed by refluxing under CO_2 with 6 N HCl for 24 hours.

The stability of tryptophane to hot alkalies, even in the presence of cupric or ferric ions and aldehyde groups, is considerably greater than to acids. Homer (305) found that heating at 100° for 20 to 120 hours with 14 per cent baryta did not destroy tryptophane in commercial casein. On the other hand Onslow (483), Kraus (388), and Gordon (261A) reported a considerable destruction of tryptophane by hot NaOH, Na_2CO_3, and $Ba(OH)_2$. Onslow (483) noted that the quantity of tryptophane destroyed by hot baryta was less in the presence of other amino acids.

Folin and Ciocalteu (232) claimed that hydrolysis of proteins with 14 per cent baryta at 100° for 48 hours, according to Homer (305), gave low and erratic results. They found, in contrast to Homer (305), Kraus (388), Herzfeld, Fürth and others (*cf.* 462) that "Tryptophane is far more stable in alkaline solutions and less stable in acid solutions than has heretofore been recognized." They suggested boiling the protein with 5 N NaOH for 18 to 20 hours over a low flame.

As a result of Folin's experiments, hydrolysis with 5 N NaOH is generally employed. However, Jorpes (344) recovered only 78 to 98 per cent (mean 82.4 ± 2.2 per cent) of tryptophane added to gelatin after hydrolysis with 5 N NaOH at 100° for 20 hours. Webb

and Block (672) determined the comparative effects of hydrolyzing for 5 hours at 125° (oil bath temperature) with 5 N LiOH, KOH, NaOH, and 20 per cent $Ba(OH)_2$ and $Ca(OH)_2$ (suspension). Casein, egg albumin, lactalbumin, gelatin, and serum proteins were the test proteins. Recovery experiments with tryptophane were run. The results did not indicate the advantage of one alkali over another with the exception of $Ca(OH)_2$ which was definitely inferior.

Lugg (430) has reported that the presence of 5 per cent $SnCl_2$ in 5.5 N NaOH reduces the hydrolytic destruction of tryptophane in the presence of relatively large quantities of carbohydrate. When the protein is heated for 20 to 30 hours at 100° with 5 N NaOH, Lugg finds a 6 per cent loss. If the protein is hydrolyzed under nitrogen with 5 per cent $SnCl_2$ in 5.5 N NaOH the loss is reduced to 3 per cent. The use of $SnCl_2$ necessitates the removal of the Sn with zinc, a tedious procedure.

Miller and Lyons (454) on the other hand, claim that tryptophane is progressively destroyed by refluxing with 15 per cent H_2SO_4, 20 per cent HCl or 10 to 50 per cent $Ba(OH)_2$. They say that the tryptophane values, obtained on hydrolysis of a protein, represent the result of the difference between the amino acid liberated during hydrolysis and that destroyed. It should be pointed out that Miller and Lyons (454) used the color produced with NaOCl to estimate tryptophane. This method has not been generally employed.

Bolling and Block (112) found that varying quantities of tryptophane, added to lactoglobulin, were quantitatively recovered following hydrolysis with 5 N NaOH at 115 to 125° oil bath temperature. Both the Folin phenol (233) and Millon-Lugg (429) methods were employed and the analytical results were subjected to statistical analysis. However, this finding cannot be generalized as lactoglobulin is devoid or almost devoid of carbohydrate.

Phenylalanine: The chemical formula of this amino acid suggests stability. It was, therefore, somewhat of a surprise that Block, Jervis, Bolling, and Webb (105) found that casein, egg albumin, lactalbumin, gelatin, and serum proteins gave more phenylalanine after hydrolysis with 5 N NaOH than with 8 N H_2SO_4, 20 per cent HCl, HCl–HCOOH, and especially 57 per cent HI. The slight superiority of NaOH hydrolysis over acid hydrolysis was confirmed by Fontaine, Olcott, and Lowy (238) and by Knight and Stanley (370). The latter investigators made the interesting observation that the presence of relatively large quantities of tryptophane (4 to 5 per cent) in the protein results in high phenylalanine values.

Therefore, the quantity of phenylalanine found must be corrected by subtracting a value corresponding to the amount of tryptophane present in the aliquot of the solution taken for analysis.

Bolling (112) has confirmed the earlier findings that alkaline hydrolysates (5 hours) may contain slightly more phenylalanine than acid hydrolysates (18 hours). The addition of 2 and 4 per cent of phenylalanine to lactoglobulin before acid hydrolysis gave 95 and 92 per cent recoveries. However, these losses were not statistically significant (critical ratios less than 3.3). The addition of 12 per cent of the weight of the protein as phenylalanine resulted in only an 87 per cent recovery. This loss was significant (critical ratio 8.1). On the other hand, Bolling's experiments (113) indicated that when phenylalanine is heated at 110 to 115° for 5 hours with 5 N NaOH it is destroyed or so changed as not to be estimated by the Kapeller-Adler method. The amount of destruction increases as the percentage of phenylalanine in terms of protein hydrolyzed is increased. Thus, when 2 per cent of phenylalanine was added to lactoglobulin before hydrolysis, approximately 90 per cent was recovered, 4 per cent of phenylalanine gave a recovery of approximately 80 per cent, while the addition of 12 per cent of phenylalanine, resulted in the recovery of only 70 per cent. In the absence of protein, the loss was much greater. When 4 mg. of phenylalanine were heated at 110 to 115° (oil bath temperature) with 8 ml. of 5 N NaOH for 5 hours, only 25 per cent of the phenylalanine could be found.

Comment: It is apparent from the above discussion that destruction of tyrosine, tryptophane, and phenylalanine can and often does occur during hydrolysis. The amount of destruction is dependent upon the conditions in the hydrolysate, this includes strength of acid or alkali, time and temperature of hydrolysis, presence or absence of oxygen and oxygen carriers, presence of aldehydes, and the ratio of the amino acid in question to undecomposed protein and to the liberated amino acids.

It is thus unwise to suggest certain hydrolytic conditions or a constant correction factor but for greatest accuracy several methods of hydrolysis and the addition of varying quantities of the amino acids to be determined both *before* and *after* hydrolysis is advised. For routine work, 5 hour hydrolysis with an excess of 5 N NaOH at 115 to 125° usually yields satisfactory results for the aromatic amino acids.

CHAPTER II

PART II

THE DETERMINATION OF TYROSINE*ᵃ

1. Isoelectric Precipitation

Principle: The fact that tyrosine is only slightly soluble in neutral or faintly acid aqueous solutions was the basis for the isolation and identification of this amino acid and was used by the earlier protein analysts to estimate the minimal quantity of tyrosine present in a protein hydrolysate. After hydrolysis, the excess acid was removed and the amino acid solution was adjusted to neutrality with ammonia. The filtrate was concentrated and the precipitate of crude tyrosine was removed. The filtrate was further concentrated and a second crop was obtained. This process was supposed to be repeated until the mother liquor gave a negative Millon's test, a goal which, however, was seldom realized. The crude tyrosine was purified by recrystallization.

Comment: This method which gave minimal values and required large quantities of protein is rarely employed at the present time.

2. Reaction with Mercury Salts and Nitrous Acid (Millon, 456)

Historical: In 1849, M. E. Millon (456) showed that when mercury is dissolved in an equal weight of nitric acid and $4\frac{1}{2}$ equivalents of water are added, the resulting mixture which contains $Hg(NO_3)_2$, $HgNO_3$, $HgNO_2$, HNO_2, HNO_3, etc. gave a red precipitate when warmed with proteins and a stable red solution when warmed with both acid and alkaline protein hydrolysates. Hoffmann (301) independently, in 1853 showed that tyrosine gave a red color when warmed with $HgNO_2$ in HNO_3. Some years later Nasse (472) showed that when tyrosine and other phenols were allowed to remain in contact with mercuric salts for some time and then treated with a dilute solution of $NaNO_2$, the typical red color of Millon developed.

Millon's solution became popular for the qualitative detection of tyrosine in proteins and protein hydrolysates because of its sensitivity, but although it was used as the basis for the quantitative

* Recommended methods are starred.

ᵃ Only those methods which do not include the simultaneous estimation of tryptophane will be discussed in this section.

determination of phenols in industrial processes, it was not until 1919 that Weiss (675) attempted to use the Millon-Nasse reaction (HgSO₄ in H₂SO₄ plus NaNO₂) for the quantitative estimation of tyrosine in protein hydrolysates.

A. *Zuwerkalow's Modification of the Millon-Weiss Reaction* (699)

Principle: The protein is not hydrolyzed but merely dissolved in dilute NaOH.

Method: 10 mg. of protein are dissolved in 1 ml. of 5 per cent NaOH and warmed if necessary. To this solution, 3 ml. of acetic acid, 2 ml. of 10 per cent HgSO₄ in 5 per cent H₂SO₄ and 1 drop of 0.5 per cent NaNO₂ are added in order. The solution is gently warmed, but not boiled over a flame. The color is read against a tyrosine standard.

Comment: It appears to the authors that the presence of tryptophane may lead to high results.

*B. *Bernhart's Modification of the Millon-Weiss Method* (72)

Principle: The protein is hydrolyzed with NaOH, an excess of HgSO₄ in H₂SO₄ reagent is added (Hopkins and Cole, 307), and tyrosine is estimated colorimetrically in the filtrate by the addition of HONO. Tryptophane mercury complex is removed by centrifuging.

Method: 10 mg. of protein are heated with 0.2 ml. of 6 N NaOH in boiling water for 4 to 5 hours. The hydrolysate is cooled and acidified with 0.3 ml. of 7 N H₂SO₄ and 1.5 ml. of 15 per cent HgSO₄ in 5 N H₂SO₄ are added. The tube is heated for 10 minutes in boiling water. The solution is then cooled, 1 ml. of 7 N H₂SO₄ and 1 ml. of 0.2 per cent NaNO₂ are added. The solution is shaken and diluted to 10 ml. with H₂O. The precipitate of tryptophane mercury complex is removed by centrifuging. The color is read with a 520 mu filter.

Comment: Although Fürth (248) has claimed that the Millon methods for estimating tyrosine in proteins do not yield absolute values and are only useful for comparative results, it has been the authors' experience that the methods will determine with considerable accuracy the quantity of phenolic groups present in the hydrolysates of most purified proteins.

C. *Fürth and Fischer's Adaptation of the Millon Reaction* (250)

Reagents: Millon's reagent. 1 part of Hg is dissolved in 2 parts by weight of HNO₃ (sp. gr. 1.42). The solution is then diluted with 2 volumes of water.

Method: 1. Hydrolysis. 2.5 gm. of protein are hydrolyzed for 12
hours under reflux with 3.5 ml. of H_2SO_4 and.22.5 ml. of H_2O. The
solution is diluted to 50 ml.

2. Precipitation with Phosphotungstic Acid. A 20 ml. aliquot of
the hydrolysate is treated with 20 to 40 ml. of 20 per cent phospho-
24-tungstic acid. The precipitate is allowed to form for several days
before it is filtered off. The excess phosphotungstic acid is removed
from the filtrate with 5 per cent quinine sulfate in 5 per cent HCl
and the excess quinine is precipitated by making the filtrate alka-
line with NaOH.

3. Development of Color. To 10 ml. of the above solution, 2 ml.
of Millon's reagent are added. The reaction is allowed to take place
at room temperature until maximum color has developed and then
read as usual.

Comment: Leipert and Alcock (413) add $HgSO_4$ in H_2SO_4 and
$NaNO_2$ directly to the phosphotungstic acid filtrate without re-
moving the excess reagent.

3. THE DIAZO REACTION FOR TYROSINE (PAULY, 513, 514)

For the historical basis and structural formulae *cf.* Chapter I,
Part III.

A. *Hanke's adaptation of the Pauly Reaction for Tyrosine* (279)

Principle: Histidine is removed from the hydrolysate by pre-
cipitation with Ag_2SO_4 and $Ba(OH)_2$. Tyrosine is precipitated from
this filtrate with mercuric acetate and sodium chloride. The puri-
fied tyrosine solution is diazotized with the Weiss-Ssobolew reagent
(674, *cf.* Chapter I, Part III, 279, 281, 375).

$$\text{Tyrosine Mercury Complex} \qquad \begin{matrix} HgCl \cdot & HgCl \\ \diagdown & \diagup \\ \end{matrix} ?$$

$$HOC_6H_4CH_2CHNCOOH$$
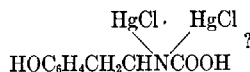
Method: Histidine is precipitated from an H_2SO_4 hydrolysate of
5 gm. of protein with Ag_2O–$Ba(OH)_2$ according to the usual Kossel
method (*cf.* Chapter I). The filtrate is immediately acidified to
prevent the loss of tyrosine by oxidation by Ag_2O. After removal
of reagents with HCl and H_2SO_4, the amino acid solution is evap-
orated to dryness. The residue is dissolved in 75 ml. of water, 1
ml. of glacial acetic acid and 3.5 gm. of mercuric acetate are added.
The solution is boiled for 10 minutes under reflux. Cooled and 7.5
gm. of NaCl are added. The precipitate of tyrosine mercuric chlo-
ride complex, after remaining in the cold for 2 hours, is removed by
centrifugation and is washed with 25 ml. of 10 per cent NaCl. The
precipitate is decomposed with hot 20 per cent HCl and the Hg

is removed with H_2S. After concentrating to dryness, the residue is taken up in water and a small aliquot is used for the determination of tyrosine by the Koessler-Hanke (375) modification of the Pauly-Weiss method (513, 674), *cf.* Chapter I, Part III, The Diazo Reaction.

Comment: The tyrosine values obtained by the diazo method were lower than those reported about the same time by Folin and Looney (231). This led to a rather acrimonious debate over the value of the Pauly, phenol, and Millon reactions for the determination of tyrosine. As a result Hanke (388) admitted that some tyrosine was lost by the Ag_2SO_4–$Ba(OH)_2$ precipitation but he also showed that the tyrosine results found by the Folin phenol method were too high.

It appears to the authors that if the histidine were precipitated with silver at *pH* 7 or with $HgCl_2$ in neutral or very faintly alkaline solution (Kossel, 378) and then the tyrosine were precipitated in acid medium (mercuric acetate-NaCl), the Pauly-Hanke method would prove to be of considerable value.

4. REACTION WITH NITROSONAPHTHOL IN NITRIC ACID (GERNGROSS, 256)

Principle: Gerngross, Voss and Herfelt (256) noticed that a red color was produced when some HNO_3 was accidentally spilt on the skin on which some 1,2,nitrosonaphthol was already present.

Method: 3 mg. of protein are dissolved by warming in 3 ml. of dilute NaOH or HCl. The solution is neutralized and 1 drop of 0.1 per cent 1,2,nitrosonaphthol followed by 6 drops or more of concentrated HNO_3 are added. The solution is boiled and the color is read against a 1:1,000,000 tyrosine standard treated in the same way.

Comment: It is claimed that the reaction is specific for p-alkylated phenols. As far as the authors are aware, this promising test has not been studied by other investigators.

PART III

THE DETERMINATION OF TRYPTOPHANE[a]

1. ISOLATION (HOPKINS AND COLE, 307)

Principle: The protein is digested with trypsin. Tryptophane is precipitated from the digest with 10 per cent $HgSO_4$ in H_2SO_4 and is isolated after decomposing the mercury complex.

Reagents: Hopkins-Cole or Denigès. 250 ml. of concentrated H_2SO_4 are added to 4,750 ml. of water, then 500 gm. of $HgSO_4$ are added in 10 fifty gm. portions until all of it has dissolved. The small residue is removed by filtration.

Comment: This method, which was used by the discoverers of tryptophane, would have little except historical interest but for their report that 1.5 per cent of tryptophane was isolated from casein. This value is equal or higher than many colorimetric results and has been exceeded by only one investigator, Dakin (183), who isolated 1.7 per cent of tryptophane from casein.

In both of these cases commercial trypsin was used. Hopkins and Cole (307) used 400 ml. of liquor pancreaticus per kg. of casein and Dakin (183) employed an unstated quantity of pancreas extract. It seems reasonable to assume that during these long digestions (7 to 14 days) a considerable proportion of the pancreatic proteins were also hydrolyzed. The tryptophane so liberated would have been calculated as derived from casein. The presence of 1.4 per cent of tryptophane in pancreas proteins may account for the fact that more tryptophane was actually isolated from "casein" than has been shown to be present by some of the more accurate colorimetric methods. In an attempt to clear up this discrepancy, Shaw and McFarlane (577) were able to isolate less than 1 per cent of tryptophane in casein after tryptic digestion. A value which was but slightly lower than found colorimetrically.

2. REACTION WITH GLYOXYLIC ACID (HOPKINS AND COLE, 306)

Historical: The beautiful demonstration in 1901 by F. G. Hopkins and S. W. Cole (306) that glyoxylic acid was the essential component in the glacial acetic acid test of Adamkiewicz is too well known to warrant elaboration.

[a] Only those methods which do not include the simultaneous estimation of tyrosine will be discussed in this section.

A. Cary's Application of the Hopkins-Cole Method (149)

Principle: Tryptophane is precipitated with mercuric sulfate in H_2SO_4. The precipitate is suspended in strong H_2SO_4 and glyoxylic acid is added.

Reagents: Glyoxylic Acid Reagent. 100 ml. of saturated oxalic acid are reduced with 6 gm. of 5 per cent Na–Hg amalgam. One ml. of the clear filtrate is added to a mixture of 20 ml. of diluted H_2SO_4 (21 ml. of H_2SO_4 plus 5 ml. of H_2O).

Method: Tryptophane is precipitated with 15 per cent $HgSO_4$ in 5 per cent (by volume) of H_2SO_4. The precipitate is suspended in 20 ml. of glyoxylic acid reagent. Four drops of 25 per cent $HgSO_4$ in 10 per cent H_2SO_4 are added and the color is allowed to develop at room temperature for 48 hours. It is read against a standard.

Comment: Like other tryptophane tests, this reaction is given by indols.

B. Brice's Use of the Glyoxylic Acid Method (132)

Method: To 1 ml. of unknown add 1 ml. of aqueous glyoxylic acid and 3 ml. of concentrated H_2SO_4. The latter should be added slowly with shaking. Stand several minutes, cool, and read. Use tryptophane as the standard.

C. Winkler's Modification of the Hopkins-Cole Procedure (682)

Principle: Small amounts of copper sulfate greatly enhance the amount of color given by the Hopkins' glyoxylic acid method.

Reagent: Glyoxylic Acid according to Benedict (61). To 10 gm. of powdered Mg, add sufficient water to cover the same. Then add 250 ml. of cold saturated oxalic acid. Cool under the tap during slow addition. When the reaction has ceased, remove the magnesium oxalate. Wash the precipitate with a little water, acidify the filtrate and washing with acetic acid and dilute to 1 liter.

Method: Dissolve 10 mg. of protein in HCOOH or NaOH and dilute to a convenient volume. To 0.1 to 2.5 ml. of unknown, containing 0.005 to 0.150 mg. of tryptophane, add with agitation 0.50 ml. of glyoxylic acid-copper sulfate (2 parts of Benedict's glyoxylic acid plus 3 parts of $M/100$ $CuSO_4$). Dilute with water to 3 ml., cool to $-10°$ and add 5 ml. of cold concentrated H_2SO_4 at such a rate that the solution does not get warm. Stand at room temperature at least 3 to 4 hours, then heat in a boiling water bath for 5 minutes and read the color with 530 mu filter.

Comment: Small quantities of $CuSO_4$ increase the sensitivity of the glyoxylic acid test from 1:200,000 to 1:1,000,000. The presence of traces of copper in the reagents may explain previous difficulties with the Hopkins-Cole methods.

*D. *Shaw and MacFarlane's Modification of the Hopkins-Winkler Procedure* (576).

Reagents: Glyoxylic Acid. Treat 100 ml. of 5 per cent aqueous oxalic acid with 3.0 ml. of N/5 $HgCl_2$ and a few pieces of Al wire. Place in a boiling water bath for 5 minutes after the appearance of bubbles on the Al wire. Stand at room temperature for 5 minutes, filter, and add 2.0 ml. of H_2SO_4. Keep cold.

Method: 1. Hydrolysis. Dissolve 10 mg. of protein in 10 or 20 per cent NaOH by warming in 5 per cent formic acid, or by the usual hydrolysis with 5 N NaOH or saturated baryta.

2. Development of Color. To 0.1 to 2.0 ml. of unknown, containing 0.005 to 0.150 mg. of tryptophane, add 0.50 ml. of glyoxylic acid and 0.50 ml. of M/25 $CuSO_4$. Dilute to 3 ml. Cool thoroughly in an ice bath. Add 0.5, 1.0, 1.5, and 2.0 ml. portions of concentrated H_2SO_4 from a burette with cooling between each addition. Stand at room temperature for 10 minutes, heat in boiling water for 5 minutes, cool and dilute to 10 ml. with 5:3 (by volume) of H_2SO_4. Read in 15 minutes using both filters 540 mu and 520 mu. Each reading should check its respective curve; if not, interfering substances are assumed to be present.

Comment: Hopkins and Cole (306) pointed out that H_2O_2 interferes with the tryptophane-glyoxylic acid reaction.

3. REACTION OF TRYPTOPHANE WITH ALDEHYDES (VOISENET, RHODE, 665, 545)

Historical: Following the demonstration of Hopkins and Cole (306) in 1901 that tryptophane gave a color with glyoxylic acid in the presence of H_2SO_4, Voisenet (665), in 1905, showed that proteins, indole, skatol, etc. gave highly colored solutions when treated with KNO_3, concentrated HCl and various aliphatic and aromatic aldehydes. Voisenet used HCHO, CH_3CHO and its polymers, chloral, higher aliphatic aldehydes including glucose, C_6H_5CHO, HOC_6H_4CHO and other aromatic aldehydes. Concentrated H_2SO_4 can be used in place of HCl.

Rhode (545), in the same year, reported that proteins gave a red color with 5 per cent p-dimethylaminobenzaldehyde in 10 per cent H_2SO_4 when concentrated HCl was added. Other aromatic aldehydes such as vanillin (m-methoxy, p-hydroxybenzaldehyde) and p-nitrobenzaldehyde can be used in place of Ehrlich's reagent.

Numerous investigators have used the Voisenet-Rhode reactions as the basis for the determination of tryptophane in proteins and indoles in biological material.

A. *Thomas's Application of the Voisenet-Rhode Reaction* (608)

Principle: The protein is hydrolyzed with trypsin before treatment with the color reagent.

Method: 1. Hydrolysis. 400 mg. of protein are dissolved in 200 ml. of 0.5 per cent Na_2CO_3 and digested with 100 mg. of pancreatin at 37° until the maximum quantity of tryptophane is liberated as indicated by the HOBr–HBr test, 5 to 7 days.

2. Development of Color. The digest is filtered. A 50 ml. aliquot is treated with 10 ml. of 2 per cent p-$(CH_3)_2N \cdot C_6H_4 \cdot CHO$ in 20 per cent HCl and made up to 100 ml. with concentrated HCl. The solution is allowed to stand for 48 hours and compared with a standard prepared in the same way and allowed to stand under the same conditions of light and temperature.

B. *May and Rose's Modification of the Voisenet-Rhode Reaction* (440)

Method: 50 to 100 mg. of protein are weighed into a flask and exactly 100 ml. of 1:1 HCl, containing 1 ml. of 5 per cent p-dimethylaminobenzaldehyde in 10 per cent H_2SO_4, is added. The solution is placed at 35° for 24 hours and then at room temperature for 40 hours longer. The blue color is compared with casein treated in the same way. Casein is assumed to have 1.5 per cent of tryptophane.

Comment: Holm and Greenbank (303) warm at 37° for 8 to 10 days and compare with the color produced by tryptophane standards.

C. *Fürth and Dische's Modification of the Voisenet Reaction* (249)

Method: Treat 2 ml. of solution containing 2 mg. of protein in 30 per cent KOH with several drops of 2.5 per cent HCHO and add 15 ml. of pure HCl (sp. gr. 1.175). Mix, wait 10 minutes and add 10 drops of 0.05 per cent $NaNO_2$ and then concentrated HCl to 20 ml. Filter and read. Add a few drops more of $NaNO_2$ to be sure that maximum color has been achieved.

*D. *Kraus's Adaptation of the Voisenet-Rhode Reaction* (388, 389)

Principle: The protein is hydrolyzed, any indole or skatole which may have been formed during the hydrolysis is removed by extraction with toluene, and the tryptophane is precipitated with mercuric sulfate. Vanillin is used as the color reagent.

Method: 1. Hydrolysis. The protein is hydrolyzed with 14 per cent baryta at 100° (boiling water) for 40 hours or with pancreatin (*cf.* Thomas, 608) for 5 days.

2. Precipitation. An aliquot of the hydrolysate, containing 0.2 to 1.0 mg. of tryptophane is precipitated with 15 per cent $HgSO_4$ in 5 per cent H_2SO_4. After standing for 2 hours, the precipitate is removed by centrifuging and washed with Denigès' reagent. The precipitate is suspended in 1 ml. of 2 per cent $HgSO_4$, 0.4 ml. of 0.5 per cent vanillin in 50 per cent acetic acid, and 12 ml. of concentrated HCl are added. The color is read after 24 hours.

Comment: The reaction can be carried out directly on the protein hydrolysate. Kraus (388) reported that the loss during baryta hydrolysis was 20 per cent by the Voisenet-Rhode procedure and 7 per cent by the Folin phenol method. On the other hand, Fürth and Dische (249) have claimed that tryptophane is very stable to alkaline hydrolysis in the presence of other amino acids.

In cases where the tryptophane is low 0.2 mg. of the amino acid are added before $HgSO_4$ precipitation. If the tyrosine concentration is high relative to tryptophane, it may be precipitated to a certain extent by $HgSO_4$. It is claimed that NaCl interfered with the precipitation of tryptophane by mercuric sulfate, the authors have not been able to substantiate this report.

E. *Komm's Adaptation of the Voisenet-Rhode Reaction* (377)

Principle: Aldehydes condense with free tryptophane and tryptophane in proteins at different rates. It is, therefore, undesirable to use free tryptophane as a standard in the Voisenet-Rhode methods unless a tryptophane-free protein such as gelatin is used in preparing the standard.

Method: 20 to 50 mg. of protein are suspended in 2 ml. of water. 2 ml. of *either* 0.25 per cent $(CH_3)_2N \cdot C_6H_4 \cdot CHO$ in 10 per cent HCl *or* 3.75 per cent of HCHO in 10 per cent HCl are added. Then 5 or 6 ml. of 10 per cent HCl and 1 ml. of 5 per cent gelatin in 10 per cent HCl are added. 10 ml. of concentrated H_2SO_4 are run down the side of the tube and carefully mixed with the aqueous layer. The solution is cooled, after 20 minutes, the color is read against a tryptophane standard treated in the same way.

Comment: The values with *both* aldehydes should give closely checking results.

F. *The Voisenet-Rhode Reaction as Used by Tomiyama and Shigematsu* (614)

Method: Dissolve 50 mg. of protein (casein) in 2 ml. of 0.2 per cent NaOH at 50°, cool and add 100 ml. of 19 per cent HCl and 1 ml. of 5 per cent p-dimethylaminobenzaldehyde in 10 per cent H_2SO_4. Incubate at 30° until maximum color. Treat tryptophane

in the same way to prepare a calibration curve or to standardize a solution of molybdenum blue.

G. *The Bates Adaptation of the Voisenet-Rhode Method* (54)

Method: Dissolve 50 mg. of protein (casein) in 2 ml. of 0.1 N NaOH, and add 0.5 ml. of 5 per cent dimethylaminobenzaldehyde, 0.2 ml. of 1 per cent NaNO₂, and 25 ml. of concentrated HCl. Stand 15 minutes and dilute to 50 ml. with water. Read after 15 minutes.

H. *Sullivan, Milone, and Everitt's Modification of the Voisenet-Rhode Reaction* (599)

Method: Place 100 mg. of casein in a 250 ml. Erlenmeyer flask and add 99 ml. of 17.5 per cent HCl and 1 ml. of 5 per cent p-dimethylaminobenzaldehyde in 10 per cent H_2SO_4. Maintain the solution at 85° for 15 minutes. Then introduce 0.3 ml. of 0.3 per cent of H_2O_2. Shake. Cool and read against a standard prepared from casein by May and Rose's (440) adaptation of the Voisenet-Rhode reaction.

COMMENT ON ALDEHYDE METHODS FOR TRYPTOPHANE

The large number of methods which have been·proposed for the quantitative determination of tryptophane in proteins based upon the reactions of Hopkins and Cole and Voisenet and Rhode, indicate that these methods give promise of simplicity and accuracy without, however, completely achieving this goal.

Voisenet (665) pointed out that oxidizing agents (KNO_3) increased color formation while Thomas (608) showed the importance of carrying out .the reaction under carefully controlled conditions, not only with respect to the reagents used but to the temperature and quantity of light to which the reaction mixture was exposed during the development of color. Fürth and Dische (249) showed that the production of color was a function of the quantities of water and nitrous acid present as well as the presence or absence of a protective colloid. They claimed that small changes in the H_2O content of the reaction mixture had a much greater destructive action on the blue color developed in the absence of colloids than in their presence. This would give the illusion of more tryptophane in the intact protein than after hydrolysis.

Rapoport and Eichinger (538) showed that the Voisenet color varies with the strength of the NaOH used to dissolve the protein and with the concentration of HCHO used. Boyd's careful investigation (117) of the Voisenet-Rhode methods showed that color

formation was a function of the quantities of oxidizing ($NaNO_2$, $NaNO_3$, H_2O_2, etc.) and reducing (HCHO, glucose, etc.) substances present in the reaction mixture, to exposure to light, to the presence of toluene and other chemicals, etc. The total color formed at any time is the resultant of increasing color with time minus the amount of fading. Boyd (117) found a further complicating factor, different proteins produced colors of different tints.

Shaw and McFarlane (577) in 1940 compared the Voisenet-Rhode reaction (p-dimethylaminobenzaldehyde with H_2O_2 as the oxidizing agent) with the Hopkins-Cole glyoxylic acid method. Tryptophane was used as the standard. Their results follow:

Substance	TRYPTOPHANE		
	Voisenet-Rhode		Hopkins-Cole
	24 hr. at 37° per cent	15 min. at 85° per cent	(cf. 576) per cent
Casein	2.4	2.5	1.2
Casein hydrolyzed	1.3	1.9	1.2
Edestin	2.6		1.3
Tryptophane	1.0	1.5	1.0
Tryptophylglycine	1.3	2.0	1.0
Glycyltryptophane	1.4	2.0	1.0
Acetyltryptophane	1.4	2.0	1.0

In conclusion it should be pointed out that all these aldehyde tests are for the indole ring and are not specific for tryptophane except in the absence of other indole compounds.

4. OTHER COLORIMETRIC TESTS FOR TRYPTOPHANE

Historical: The formation of colored compounds when indole derivatives are treated with oxidizing agents ($CaOCl_2$, KNO_2, $FeCl_3$, $CuSO_4$, etc.) in acid solution has been known since the studies of Jaffé, Obermayer, Salkowski, and others on urinary indican, indoxylsulfuric acid. Jolles (337) showed that indican gave a strong stable color when treated with Obermayer's reagent ($FeCl_3$ in HCl) in the presence of thymol or other phenols. Many of these indole reactions have been reviewed by Homer (304).

A. *Miller and Lyons' NaOCl Reaction* (453, 454)

Procedure: 5 ml. aliquots of a neutralized protein hydrolysate, containing about 0.7 mg. of tryptophane are treated with 0.2 to 1.8 ml. of 0.15 per cent NaOCl in 0.2 ml. increments. Then 5 or 10 drops of 0.4 N-HCl are added and the tubes with the most color are immediately extracted with isoamyl alcohol. The color is read against a tryptophane standard prepared in the same way.

Comment: Miller and Lyons originally used Engel's NaOCl–C_6H_5OH test and later observed that the reaction took place as well in the absence of carbolic acid or other phenols.

B. *Albanese and Frankston's Adaptation of the Jolles Test* (28)

Method: 1. Hydrolysis. Hydrolyze 1 gm. of protein for 20 to 22 hours with 5 ml. of 20 per cent NaOH. Neutralize to *p*H 7 with acetic acid and dilute so that 1 mg. of tryptophane is present in each 2 ml. of solution.

2. Development of Color. To 2 ml. of hydrolysate, add 0.3 ml. of 3 per cent $NaNO_2$, 0.1 ml. of 10 per cent acetic acid. Shake intermittently for 10 minutes, add 0.3 ml. of 1 per cent $K_2S_2O_8$, 0.5 ml. of 1 per cent thymol in 95 per cent ethanol and 5 ml. of a mixture of 3 parts of 40 per cent trichloracetic acid and 2 parts of concentrated HCl. Mix after each addition. Place in a boiling water for 5 minutes, cool in an ice bath for 5 minutes, remove the colorless aqueous layer by a pipette, dilute the colored layer to 5 ml. with glacial acetic acid and read using 540 mu filter.

Comment: The Miller-Lyons and Jolles-Albanese methods, like the aldehyde procedures, are not specific for tryptophane, but give colored products with other indole containing compounds.

C. *Nicol's Reaction* (480)

Principle: Tryptophane is treated with nitrous acid and the resulting product is condensed with N(1-naphthyl)-ethylenediamine to give a red color.

Reagents: 0.1 per cent N(1-naphthyl)-ethylenediamine dihydrochloride is prepared in a dark bottle and kept in a cool place.

Method: Add 1 ml. of 1 per cent $NaNO_2$ to 5 ml. of the hydrolysate containing 0.1 to 0.3 mg. of tryptophane in 1.2 N HCl. Stand 30 minutes. Swirl occasionally. Add 4 ml. of 4 per cent ammonium sulfamate, mix, and stand 10 minutes. Then add 10 ml. of water and 5 ml. of the color reagent. Stand 30 to 60 minutes. Read using a 560 mu filter.

Comment: If interfering colors are present, the red dye can be extracted after maximum color development by adding 10 ml. of n-butyl alcohol and 5 gm. of NaCl to the solution. Shake thoroughly. Filter the butanol into a clean reading tube. Keep conditions constant.

PART IV

THE DETERMINATION OF TYROSINE AND TRYPTOPHANE

1. THE MILLON-FOLIN REACTIONS (231, 232, 233)

Historical: In 1922, Folin and Looney (231) used the Denigès-Hopkins mercuric sulfate reagent to separate tryptophane from the other amino acids of a protein hydrolysate. The tyrosine which remained in the filtrate was then estimated by employing the Millon-Nasse reaction (456, 472) while the quantity of tryptophane in the precipitate was determined by its reducing action on phosphomolybdic acid (231) in alkaline solution. Later phosphomolybdotungstic acid was used (232).

A. *The Method of Folin and Marenzi (233)*

Reagents: Phosphomolybdotungstic acid (Phenol Reagent). 100 gm. of $Na_2WO_4 \cdot 2H_2O$ and 25 gm. of $Na_2MoO_4 \cdot 2H_2O$ are dissolved in 700 ml. of H_2O. 50 ml. of 85 per cent H_3PO_4 and 100 ml. of concentrated HCl are added and the solution is boiled under reflux for 10 hours. At the end of this time, 150 gm. of Li_2SO_4, 50 ml. of H_2O and a few drops of Br_2 are added. The solution is boiled without the condenser for 15 minutes to remove the excess bromine. After cooling, it is diluted to 1 liter, filtered and kept in a brown bottle.

15 per cent Mercuric Sulfate in 6 N H_2SO_4: 150 gm. of $HgSO_4$ are suspended in 400 to 450 ml. of 7 N H_2SO_4. 150 ml. of H_2O are added and the suspension is shaken until completely dissolved. The solution is diluted to 1 liter with 7 N H_2SO_4, filtered if necessary.

1.5 per cent Mercuric Sulfate: To 100 ml. of 15 per cent $HgSO_4$ reagent, 100 ml. of 14 N H_2SO_4 are added and the solution is diluted with water to 1000 ml.

14 N Sulfuric Acid: 392 ml. of concentrated H_2SO_4 are diluted to 1 liter with water.

Saturated Na_2CO_3: do not allow this reagent to come in contact with rubber.

Method: 1. Hydrolysis. Folin and Looney (231) boiled the protein with 14 per cent baryta for 40 to 48 hours under reflux. Folin and Ciocalteu (232) hydrolyzed with 5 N NaOH under reflux for 18 to 20 hours. Folin and Marenzi (233) heated the protein solution with 5 N NaOH at 100° for 12 to 18 hours. von Deseö (192) auto-

claved the protein with 5 N NaOH at 2 to 8 atmospheres for 2 hours. Lugg (430) hydrolyzed with 5.5 N NaOH which contained 5 per cent $SnCl_2 \cdot 2H_2O$. The hydrolysis was carried out in a sealed tube under nitrogen or in the absence of air for 20 hours at 100°. McFarlane and Fulmer (447) used pepsin and trypsin or trypsin alone in lieu of alkaline hydrolysis. This method is to be especially recommended when impure proteins such as foods and feeds are being analyzed.

It is customary to employ 2 ml. of 5 N NaOH for every 100 mg. of protein. The hydrolysate is acidified with 3 ml. of 7 N H_2SO_4 for each 2 ml. of 5 N NaOH used. The hot solution is transferred to a 25 ml. graduated cylinder and 200 to 500 mg. of kaolin or filter aid are added. The suspension is shaken well and filtered. 20 ml. of the filtrate are used for each determination.

2. Separation of Tryptophane and Tyrosine. 20 ml. of the acidified hydrolysate are placed in a 50 ml. conical centrifuge tube and 4 ml. of 15 per cent $HgSO_4$ reagent are added. The flask is set aside for 2 or 3 hours and centrifuged for 5 to 10 minutes. The filtrate is poured into a 100 ml. volumetric flask and the edge of the centrifuge tube is washed with 1 ml. of 0.1 N H_2SO_4. The tryptophane mercury precipitate is washed with 10 ml. of 1.5 per cent $HgSO_4$ reagent. The precipitate is stirred and after standing 10 minutes, it is centrifuged. The lip of the tube is rinsed with 0.1 N H_2SO_4.

3. Determination of Tyrosine. 6 ml. of 7 N H_2SO_4 are added to the filtrate which is heated in a boiling water bath for 5 minutes, cooled, and 1 ml. of 2 per cent $NaNO_2$ is added with shaking. After 2 minutes the solution is diluted to 100 ml., and read against a standard prepared simultaneously.

4. Determination of Tryptophane. 10 ml. of N HCl are added to the tryptophane mercury precipitate which is heated in boiling water for 30 minutes, cooled, and filtered into a 100 ml. volumetric flask. The solution is diluted to 60 ml., 25 ml. of cold saturated Na_2CO_3 are added, followed by 5 ml. of phenol reagent. After standing for 30 minutes, the excess reagent is destroyed with 2 or 3 ml. of 5 per cent NaCN. The solution is diluted to 100 ml. and compared against a standard prepared at the same time.

*B. *Block and Bolling's Adaptation of the Millon-Folin Method* (95)

Principle: This procedure is based on the Folin-Marenzi (233) and Bernhart (72) adaptations of the Millon-Nasse reaction.

Reagents: The Folin mercuric sulfate and phenol reagents are used (*cf.* above).

Method: 1. Hydrolysis. 100 to 500 mg. of protein are hydrolyzed with 2 to 10 ml. of 5 N NaOH under reflux for 4 to 5 hours. The temperature of the oil bath is maintained at 110 to 125°C. The alkali is neutralized with 3 to 15 ml. of 7 N H_2SO_4 respectively. The condenser is rinsed down with a little water and the hydrolysate is transferred to a graduated cylinder, diluted to volume, and filtered if necessary.

2. Separation of Tyrosine and Tryptophane. Aliquots containing approximately 0.3 mg. and 0.6 mg. of tyrosine are pipetted into 40 ml. graduated centrifuge tubes. Water is added to the 20 ml. mark. Then 6 ml. of 15 per cent $HgSO_4$ reagent (Folin) are added and the tubes are placed in a boiling water bath for 10 minutes. The solutions are cooled, 4 ml. of 7 N H_2SO_4 are added, and the solutions are diluted to 40 ml. with water. 10 to 20 mg. of diatomaceous earth (Celite-Johns Manville) are added, the suspensions are mixed and centrifuged for 5 minutes. The filtrates are poured into Evelyn reading tubes. The tryptophane precipitates are washed by centrifuging with 5 ml. portions of 1.5 per cent $HgSO_4$ in 6 N H_2SO_4 (Folin). The filtrates are added to the proper reading tubes.

3. Determination of Tyrosine. The reading tubes are warmed to 30° in a water bath and 1 ml. of 0.8 per cent $NaNO_2$ is added to each tube. The solutions are mixed and read after 10 minutes. Filter 520 mu is used with water or the entire solution without nitrite as the blank. A calibration curve over the range 0.15 to 0.80 mg. of tyrosine is prepared.

4. Determination of Tryptophane. The tryptophane mercuric sulfate precipitates are decomposed by suspending them in 3 ml. of N HCl and heating in boiling water for 10 minutes. The suspensions are cooled and the solutions are filtered through soft paper into 25 ml. stoppered graduates. The centrifuge tubes and the filter papers are washed with small portions of water until the volume of each tryptophane solution is approximately 15 ml. Six ml. of saturated Na_2CO_3 are added to each, after mixing 1 ml. of Folin's phenol reagent (phosphomolybdotungstic acid) is added. The solutions are allowed to stand for 30 minutes. (It is usually convenient to add the $NaNO_2$ to the tyrosine solutions and determine the same during this half hour interval.) At the end of this period, 0.5 ml. of 5 per cent NaCN are added to each graduate to stop the reaction. The solutions are mixed, diluted to volume, and the colors are read against water or an hydrolysate-reagent blank with the phosphomolybdotungstic acid omitted. A 420 mu filter is used. A calibration curve over the range of 0.10 to 0.40 mg. of tryptophane should be prepared.

Comment: There have been numerous slight modifications of the Millon-Folin methods for tyrosine and tryptophane, few of which show marked improvement over Folin's own procedures and all of which give satisfactory results. In contrast to the very sensitive aldehyde methods for tryptophane, the directions for the Millon-Nasse methods for tyrosine and the Folin phosphomolybdotungstic acid methods for tryptophane can be changed considerably without harm.

Abderhalden and Siebel (23) use 7 ml. of 14 N H_2SO_4 in place of 6 ml. of 7 N H_2SO_4 recommended by Folin and Marenzi (233). von Deseö (192) uses 5 ml. of 14 N H_2SO_4 at this point to prevent turbidity. Jorpes (344) does not filter off the silicic acid after acidifying with H_2SO_4, but allows it to precipitate with the tryptophane mercuric sulfate complex. He boils the tyrosine mercury solutions for 10 to 15 minutes and then allows them to stand at room temperature for several hours. The solutions are filtered if turbid before the addition of the $NaNO_2$.

Bálint (45) uses filter 500 mu for tyrosine and 720 mu for tryptophane estimations. The Folin reagents are employed but in relatively smaller proportions, final volume 20 ml. Range, tyrosine 0.2 to 2.0 mg.; tryptophane 0.1 to 0.4 mg.

The composition and reactions of phosphotungstic and phosphomolybdic acids have been discussed by Wu (693).

Fujiwara and Kataoka (254) claim that the Folin phenol and similar reagents are non-specific, being reduced not only by phenols, but also by aromatic aldehydes, hydroxy and keto aliphatic acids, hydroxylamine, fructose, hydroxymethylfurfural, inulin, indole derivatives, morphine alkaloids, etc.

Schild and Enders (566), in a careful evaluation of the Folin tryptophane method, have shown that phosphomolybdotungstic acid gives a positive reaction, even after mercuric sulfate precipitation, with "melanoidin" formed from the condensation of glucose with glycine. Positive tests are also given by tannins, purine and pyrimidine products following alkaline hydrolysis, acetone, indole and its derivatives, pyrrol, pyruvic acid, and many other naturally occurring substances. However, the color producing ability of these compounds is not directly related to their redox potential as determined by the dichlorphenolindophenol method. Schild and Enders stress the fact that except for pure proteins and amino acid mixtures, tryptophane values by the Folin method must be accepted with caution.

2. The Millon-Nasse Reaction for Tryptophane and
for Tyrosine (456, 472, 15)

Historical: Although Abderhalden and Kempe (15) showed in 1907 that tryptophane gave a red brown color when warmed with Millon's reagent, it was not until 1937 that Lugg (429) employed the Millon reaction for the quantitative determination of this amino acid.

A. *Lugg's Use of the Millon-Abderhalden Reaction for Tryptophane* (429)

Reagents: 5 N H_2SO_4: 25 gm. H_2SO_4 per 100 ml. H_2O.

Precipitation Reagent: dissolve 75 gm. $HgSO_4$, 55 gm. $HgCl_2$, and 70 gm. Na_2SO_4 in a mixture of 850 ml. of water and 125 gm. of H_2SO_4 (68 ml. H_2SO_4, sp. gr. 1.84). Dilute to 1 liter.

Washing Reagent: dilute the Precipitation Reagent with an equal volume of N H_2SO_4.

Color Reagent: dissolve 12 gm. of $HgSO_4$ and 9 gm. of $HgCl_2$ in a mixture of 600 ml. of H_2O and 100 gm. of H_2SO_4 (54 ml. H_2SO_4, sp. gr. 1.84), then add 500 gm. more of H_2SO_4 (270 ml.). Cool, dilute to 1 liter.

Method: 1. Hydrolysis (430). Hydrolyze 100 mg. of protein with 2 ml. of either 5 N NaOH or 5.5 N NaOH containing 5 per cent of $SnCl_2 \cdot 2\ H_2O$ at 100° for 20 to 24 hours.

If alkaline stannite is used, it is advisable to remove the interstitial air from the protein by evacuation and to let in the alkali under vacuo. At the end of the hydrolysis, the tin is removed by shaking three times with 200 mg. portions of zinc dust per 10 ml. of reagent used. The precipitates are separated by centrifuging and washed with 0.1 N NaOH. The combined alkaline solutions are extracted with 1 or 2 volumes of ether or toluene according to Kraus (388) to remove indole, skatole, phenols, etc.

The alkaline solutions are acidified with 5 ml. of 15 N H_2SO_4 per 10 ml. of NaOH reagent used. The acidified solution is clarified by centrifugation and the hydrolysate is again extracted with 1 to 2 volumes of ether. The solvent is washed with a little water. The dissolved ether is removed at 45° or less.

2. Separation of Tryptophane from Tyrosine. To 3 ml. of unknown, add 5 N H_2SO_4 to pH 0.3 (brilliant cresyl blue), then N H_2SO_4 to 5 ml. To this solution, add 5 ml. of Precipitation Reagent and place in a water bath at 60 to 65° for 30 minutes. Cool 1 hour at 18 to 20°, centrifuge and wash once with 10 ml. of Washing Reagent.

3. Determination of Tyrosine. The filtrates are diluted to 24.5 ml. in a graduated cylinder and 0.5 ml. of 6.9 per cent m NaNO₂ are added, the solutions are mixed and read.

4. Determination of Tryptophane. The precipitate of tryptophane mercury is suspended in 10 ml. of Color Reagent and maintained in a water bath at 40 to 45° for 15 minutes. The tubes are cooled to 18 to 20° for 30 minutes, and the precipitate is removed by centrifuging. The filtrate is poured into a 25 ml. graduated cylinder. The precipitate is again suspended in 10 ml. of Color Reagent, well stirred, and centrifuged. The combined supernatant liquids are diluted to 24.5 ml. and 0.5 ml. of m NaNO₂ are added, the solutions are mixed and read at maximum color.

Comment: Lugg (430) suggests a 3 per cent correction for tryptophane if alkaline stannite is used in the hydrolysis and one of 6 per cent when NaOH alone is employed. It appears to the authors that the correction, if any, to be used must depend on the time and temperature of hydrolysis and especially on the protein under analysis.

B. Block and Bolling's Modification of the Millon-Lugg
Method (95)

Reagents: The Lugg mercuric sulfate and chloride reagents are used. (*cf.* above).

Method: 1. Hydrolysis. Carry out with 2 ml. of 5 N NaOH per 100 mg. of protein in an oil bath at 110 to 125° for 5 hours. Neutralize the hydrolysate with 3 ml. of 7 N H_2SO_4 for each 2 ml. of 5 N NaOH used. Dilute to a convenient volume. Filter with the aid of kaolin if necessary.

2. Separation of Tyrosine and Tryptophane. Pipette aliquots containing approximately 0.3 mg. to 0.6 mg. of tyrosine into 40 ml. graduated centrifuge tubes. Add water to the 20 ml. mark. Then add 5 ml. of Lugg's $HgSO_4$-$HgCl_2$-Na_2SO_4 Precipitation Reagent and place the tubes in a boiling water bath for 10 minutes. Cool the solutions and add 4 ml. of 7 N H_2SO_4. Dilute to 30 ml. with water and add 10 to 20 mg. of diatomaceous earth (Celite-Johns Manville). Mix and centrifuge for 5 minutes. Pour the filtrates into Evelyn reading tubes. Wash the precipitates with 10 ml. of Lugg's Washing Reagent. Centrifuge and combine the $HgSO_4$-$HgCl_2$-Na_2SO_4 filtrates.

3. Determination of Tyrosine. Warm the reading tubes to 30° in a water bath and add 1 ml. of 0.8 per cent NaNO₂ to each tube. Mix and read after 5 minutes. Read the color against water or a hydrolysate-reagent blank with the NaNO₂ omitted. Use filter

520 mu. Prepare a calibration curve over the range of 0.15 to 0.80 mg. of tyrosine.

4. Determination of Tryptophane. Suspend the washed tryptophane mercury precipitate in 10 ml. of Lugg's Color Reagent (HgSO$_4$-HgCl$_2$) and place the centrifuge tube in a 50 to 55° water bath for 15 minutes. Centrifuge. Pour the supernatant solution into an Evelyn reading tube. Wash the precipitate with 10 ml. of Color Reagent and, after centrifuging, add the clear liquid to the reading tube. Add 4 ml. of water. Put the reading tube in the photoelectric colorimeter and set the galvanometer to 100. Then add 1 ml. of 3.45 per cent NaNO$_2$, mix the solution by inverting and note the maximum galvanometer deflection. Use filter 420 mu. In this way each tube is read against its own hydrolysate reagent blank. However, because the color fades almost immediately after the addition of·NaNO$_2$, the tubes should be stoppered, inverted, and read within 30 seconds.

Comment: The tryptophane results obtained by the Millon-Lugg method on hydrolysates of impure protein preparations should be accepted with reserve. It has been the authors' experience that erratic tryptophane values were obtained with certain corn proteins which were first digested with pepsin-HCl and then hydrolyzed with·5 N NaOH in the usual fashion.

The opinion is widely held that chloride interferes with the precipitation of tryptophane by mercuric sulfate and in the Millon test for tyrosine. It has been our experience that considerable quantities of chloride (NaCl) in protein hydrolysates do not necessarily interfere with either tryptophane or tyrosine determinations when they are carried out by the Millon-Lugg methods.

3. Spectrophotometric Methods for Tyrosine and Tryptophane (Holiday, 302)

Principle: Tyrosine and tryptophane in dilute NaOH have different absorption maxima in the ultraviolet region.

Apparatus: The Hilger Medium Quartz Spectrograph E 316 was used.

Method: The protein was dissolved in 0.1 N NaOH and the extinction coefficients were measured at two wave lengths. Tyrosine and tryptophane were calculated as follows:

M tyrosine $= 1.0\ E_{305} - 0.092\ E_{280}$

M tryptophane $= 0.21\ E_{280} - 0.288\ E_{305}$

where

M is the molar concentration

and E is the extinction coefficient

Comment: In view of the probable losses of amino acids during hydrolysis and other chemical manipulations, it is apparent that the estimation of amino acids in the intact or but slightly changed protein molecule is the ultimate goal. Yet, in spite of considerable promise, the spectrophotometric methods, which can only be carried out using highly specialized equipment, yield results that to date, do not appear to be superior to the chemical procedures.

COMMENTS ON TYROSINE AND TRYPTOPHANE ANALYSES

Although the specific advantages and deficiencies of the various methods have been briefly commented upon earlier in this Chapter, no mention has been made of their relative value. It is a pity that only a few investigators have made use of more than one method when analyzing for a specific amino acid. The answer, of course, is obvious for once one technique has been mastered the investigator seldom wishes to master a second or a third procedure just to "confirm" his own results. However, in those instances where one investigator has used several methods, rather interesting results can be seen.

Tyrosine: Hanke (281) claims that the Pauly diazo method checks the Millon procedure when the former is properly carried out. While Holiday (302) and Devine (195) have shown that the Millon-Folin and the spectrophotometric methods give concordant results for tyrosine in casein, blood proteins, etc.

Tryptophane: In contrast to the agreement of the Pauly, Millon, and spectrophotometric methods for tyrosine, there is no little disagreement between the values for tryptophane by the different methods. Only a few of the more recent comparisons will be given; the more detailed figures can be seen in the Tables at the end of this Chapter.

Holiday (302) claimed rough agreement between the tryptophane contents of serum albumins, globulins, and casein as determined by the Folin phosphomolybdotungstic acid method and by spectrophotometry. In contrast, Devine (195), in an excellently conceived study on Bence-Jones protein, reported 1.4 per cent of tryptophane by the Folin method and 2.5 per cent of this amino acid by the spectrophotometric procedure.

Li, Lyons, and Evans (419) found the tryptophane content of pituitary lactogenic hormone to be 1.3 per cent by the Millon-Lugg reaction and 2.5 per cent by the Hopkins-Shaw procedure. Analogous discrepancies have been reported in the tryptophane content of tobacco mosaic virus by Ross (556). This investigator found 2.0 per cent of tryptophane by the Millon-Lugg reaction

and 4.5 per cent of tryptophane by the Hopkins-Shaw glyoxylic acid procedure. Knight (*cf.* 556) confirmed these methodical differences.

McFarlane and Fulmer (447) reported the tryptophane content of casein to be 1.4 per cent by the Folin method and 2.4 per cent by the Rhode-Komm p-dimethylaminobenzaldehyde procedure. If the casein were hydrolyzed by pepsin and then by trypsin instead of with NaOH, the Folin phenol reagent indicated 1.3 per cent tryptophane, a value which was confirmed by the Voisenet-Kraus vanillin method.

On the other hand, McFarlane, Fulmer, and Jukes (448) obtained reasonably concordant values for the tryptophane in egg white by the Folin phosphomolybdotungstic acid, the Rhode-May p-dimethylaminobenzaldehyde, and by the Voisenet-Kraus vanillin procedures. However, it appears that McFarlane did not consider any of these three methods to be entirely satisfactory or to yield absolute values for some years later, Shaw and McFarlane (576, 577) carried out an investigation to improve the Hopkins-Winkler glyoxylic acid procedure.

Sullivan, Milone, and Everitt (599) reported a modification of the Rhode-May dimethylaminobenzaldehyde method by which the tryptophane content of casein was believed to be 2.4 per cent if casein were the standard but if tryptophane were the standard, then the value is "not more than 1.25 per cent."

Shaw and McFarlane (577) found 2.4 per cent of tryptophane in casein by the May and Rose modification of the Voisenet-Rhode p-dimenthylaminobenzaldehyde reaction, 2.5 per cent by the Sullivan modification, and 1.2 per cent by their own adaptation of the Hopkins-Winkler glyoxylic acid method.

Bolling and Block (112) have shown that tryptophane estimated by both the Folin phenol and Millon-Lugg methods gave identical values when applied to β-lactoglobulin but as will be seen in the tables (especially Feeds and Foods) at the end of this Chapter, it has been found, in agreement with Schild and Enders (566), etc., that the Folin tryptophane method when applied to hydrolysates of impure proteins is apt to yield high values.

In conclusion, it appears from the examples given above, that the "true" or absolute value of tryptophane may not be known for any protein but, in the absence of augmenting or interfering substances, many of the methods given will yield useful comparative data.

CHAPTER II

PART V

THE ESTIMATION OF PHENYLALANINE

1. Isolation of Phenylalanine (Fischer, 220)

Historical: Phenylalanine, which had been identified in plant proteins by Schulze, Barbieri, and Bosshard (574) in 1885, was isolated by Fischer (220) from casein in one of his first applications of the ester distillation procedure.

Method: (in outline). 1. Casein was hydrolyzed under reflux with concentrated HCl for 6 hours. Fischer (220) says the yield of esters was not increased after 36 hour hydrolysis.

2. Glutamic acid was isolated directly according to Hlasiwetz and Habermann (300).

3. The amino acids were esterified in absolute alcohol by dry HCl.

4. The HCl was removed by NaOH and K_2CO_3 at $0°$. The esters were extracted with ether.

5. The ethereal solution was dried over K_2CO_3 followed by Na_2SO_4.

6. The esters were fractionally distilled *in vacuo*.

7. Phenylalanine ester was obtained in the higher boiling fractions. Phenylalanine ester hydrochloride in contrast to the other amino acid ester hydrochlorides is soluble in ether. This fact permits its ready separation from other high boiling amino acid esters.

8. Phenylalanine was identified as the phenylisocyanate and as *the copper salt. Oxidation to phenylacetaldehyde was also used as* a delicate qualitative test.

Comment: Osborne and Jones (501) showed that only about 70 per cent of the phenylalanine present in an amino acid mixture could be recovered even by the most careful and painstaking use of Fischer's method, while Abderhalden and Weil (19) were able to recover only 55 per cent of phenylalanine from an amino acid mixture.

2. Oxidation of Phenylalanine to Benzoic Acid (Schulze-Kollmann)

Principle: According to Schulze, Barbieri, and Bosshard (574), phenylalanine yields benzoic acid and benzaldehyde on oxidation

106

with chromic acid. This principle was used by Kollmann (376) to estimate phenylalanine in protein hydrolysates.

Method: 1. Hydrolysis. The protein is refluxed with 25 per cent H_2SO_4 for 30 hours after which the hydrolysate is thoroughly extracted with ether to remove any fatty acids.

2. Oxidation. Sufficient $K_2Cr_2O_7$ and H_2SO_4 are added to the aqueous solution so that it contains 20 parts by weight of $K_2Cr_2O_7$ and 17 parts by weight of H_2SO_4. The solution is refluxed for 5 to 6 hours.

3. Extraction of Benzoic Acid. After the oxidation mixture is cooled, the benzoic acid is extracted with ether. The ether is dried over Na_2SO_4 and the solvent is evaporated off. The benzoic acid is recrystallized from hot water previously saturated in the cold with benzoic acid.

Comment: As one would surmise from the experiments of Schulze *et al.* (574), the analytical values by this method are low and irregular.

3. ESTIMATION OF PHENYLALANINE BY NITRATION AND SUBSEQUENT REDUCTION (KAPELLER-ADLER, 350)

Historical: Kapeller-Adler (350) wished to simplify the Schulze-Kollmann oxidation procedure for the determination of phenylalanine by estimating the benzoic acid according to Mohler (464). Mohler's method consists in nitrating benzoic acid with $NaNO_3$ in fuming H_2SO_4 to give 3,5-dinitrobenzoic acid according to Kapeller-Adler (350) or 3,6-dinitrobenzoic acid according to Block and Bolling (98). As will be seen below, Kapeller-Adler found that phenylalanine was not oxidized and nitrated as expected to the 3,5 or 3,6 dinitrobenzoic acid, but to 3,4 dinitrophenylalanine and other 3,4 dinitrobenzene derivatives.

A. *Estimation of Phenylalanine According to Kapeller-Adler* (350)

Method: 1. Hydrolysis. Hydrolyze 1.5 to 3.0 gm. of protein for 20 hours with 25 per cent H_2SO_4. Dilute the solution to 100 ml. and adjust to contain 10 per cent of H_2SO_4.

2. Precipitation of Histidine. Precipitate the histidine from the hydrolysate with 10 per cent phospho-24-tungstic acid in 10 per cent H_2SO_4. Avoid any excess of reagent. Wash the precipitate with dilute reagent. Volume = 200 ml.

3. Destruction of Tyrosine. Add a slight excess of 0.1 N $KMnO_4$ to 20 ml. aliquots of the histidine-free amino acid solution, which should contain 1 to 4 mg. of phenylalanine. Evaporate the solution

on the steam bath. Test from time to time to be sure that no more $KMnO_4$ will be taken up. Add more $KMnO_4$ if necessary.

4. Oxidation and Nitration. Concentrate to a thick syrup, cool, add 2 ml. of 10 per cent KNO_3 in concentrated H_2SO_4, heat on the steam bath for 20 minutes, cool and transfer into a 25 ml. stoppered graduate with 7 ml. of water. Cool to 0°.

5. Development of Color. Add 5 ml. of 15 per cent hydroxylamine hydrochloride and then dilute to the mark with concentrated ammonia. After the nitrogen has ceased to come off, place the graduated cylinder in a water bath at 40° for 5 minutes, then cool to 0° for 15 minutes and read.

Comment: Kapeller-Adler (350) proposes the following mechanism.
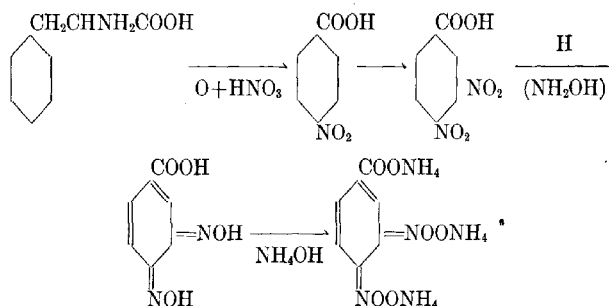
B. Kuhn and Desnuelle's Modification of the Kapeller-Adler Method (393)

Principle: The removal of histidine and the destruction of tyrosine can be omitted from the Kapeller-Adler method if the final color is read in a photometer with the proper light filter. Ascorbic acid is used as the reducing agent.

Method: An aliquot of the protein hydrolysate containing 1 to 4 mg. of phenylalanine is evaporated to dryness. 2 ml. of 10 per cent KNO_3 in concentrated H_2SO_4 are added and the nitration is carried out by warming on the steam bath for 20 minutes. The residue is dissolved in 9 ml. of water. The solution is made alkaline with ammonia, cooled, and 0.5 ml. of freshly prepared 1 per cent ascorbic acid are added. The solution is diluted with concentrated NH_4OH to 25 ml. The color is read using filter 530 mu.

Comment: The colored compound is believed to be a mixture of much *p*- and little *o*-nitrophenylhydroxylamine in the form of the mono-alkali salt.

*C. *Block and Bolling's Adaptation of the Kapeller-Adler-Kuhn Method* (104)

Method: Transfer aliquots of the hydrolysate containing approximately 0.75 mg. of phenylalanine and an equal number containing twice this amount into eight 30 cm. porcelain evaporating dishes. Evaporate to dryness on the steam bath, cool, and nitrate for 20 minutes on the steam bath with 2 ml. of 20 per cent KNO_3 in concentrated H_2SO_4. When the nitration is complete, pour the solutions into 25 ml. stoppered graduated cylinders. The final volume of each should not be over 12 ml. Cool to 0°, add 2.5 ml. of 30 per cent $NH_2OH \cdot HCl$ to three of the graduates of each set. The fourth is used as the "blank." Cool in ice. Dilute with *cold* concentrated NH_4OH to the 25 ml. mark. Swirl while adding NH_4OH. Careful! Mix and allow the color to develop at room temperature for 45 minutes. Filter if necessary before the end of the waiting period. Read against the solution to which no $NH_2OH \cdot HCl$ has been added. Color filter 560 mu. Range 0.5 to 2.0 mg. of phenylalanine.

Comment: Block and Bolling (98) report that only approximately 25 to 30 per cent of the phenylalanine present in an amino acid mixture is nitrated to the 3,4 dinitro compound. In spite of this disadvantage, the method is probably the best available at the present time excepting, of course, the highly specialized isotope procedures.

As mentioned earlier in this Chapter (Part I), the conditions of hydrolysis influence the yield of phenylalanine (*cf.* 105, 370, 238, 112).

Phenyllactic acid, if present, would be calculated as phenylalanine (321).

PART VI

CHEMICAL METHODS FOR THE ESTIMATION OF DIHYDROXYPHENYLALANINE, DIIODOTYROSINE AND THYROXINE

1. DIHYDROXYPHENYLALANINE (ARNOW, 36)

Principle: Dihydroxyphenylalanine and many other di- or triphenolic compounds give a red color in alkaline solution with nitrite.

Reagents: Nitrite-Molybdate: Dissolve 10 gm. of $NaNO_2$ and 10 gm. of Na_2MoO_4 in 100 ml. of water.

Standards: 19.2 mg. of catechol gives the same amount of color as 50 mg. of "dopa," dihydroxyphenylalanine.

Procedure: To 1 ml. of dopa solution (0.02 to 1.0 mg. of the amino acid) in a test tube graduated at 5 ml., add 1 ml. of 0.5 N HCl and 1 ml. of Nitrite-Molybdate reagent. Then add 1 ml. of N NaOH and dilute to 5 ml. with water. Read using Wratten filter 61, 500 mu, against the proper standard.

Comment: The molybdate is added to prevent too rapid destruction of HONO.

2. DIIODOTYROSINE AND THYROXINE

A. *The Determination of Thyroxine according to Blau (76)*

Principle: This is a modification of Leland and Foster's method.

Procedure: (In brief). 1. Hydrolysis. Hydrolyze 1 gm. of dry thyroid under reflux with 50 ml. of 8 per cent $Ba(OH)_2 \cdot 8H_2O$ for 6 hours. Add 2 ml. of butyl alcohol to control foaming. Cool and transfer to a 100 ml. volumetric flask. Butanol aids in the transfer. Wash the sides of the flask with 5 ml. of 10 per cent HCl. Dilute to volume.

2. Butanol Extraction. Place 50 ml. of solution in a 250 ml. separatory funnel; add 0.5 ml. of brom cresol green and titrate with 1:1 HCl to *p*H 3.4 to 4.0. Extract with an equal volume of butanol. Stand 2 hours or longer, draw off aqueous layer. Add an equal volume of 4 N NaOH containing 5 per cent Na_2CO_3. Shake, stand 1 hour or longer. Draw off aqueous layer. Reextract butanol with one half of its volume of $NaOH-Na_2CO_3$ solution. Concentrate the butanol phase to dryness. Calculate the thyroxine by an iodine determination on this residue.

B. *The Estimation of Diiodotyrosine and Thyroxine by Lugg's
    Modification of the Millon-Nasse Reaction (430, 123)*

Principle: Phenols containing halogens in the *o-* position do not
give the Millon reaction. The halogens are removed during the
hydrolysis with 5 per cent $SnCl_2 \cdot 2H_2O$ in 5.5 N NaOH at $100°$,
but not in the absence of the reducing agent (Lugg, 430).

Method: (In brief). One portion of the protein containing diiodo-
tyrosine and thyroxine is hydrolyzed with 5 N NaOH and a second
portion with 5.5 N NaOH containing 5 per cent $SnCl_2 \cdot 2 H_2O$.
"Tyrosine" is determined by the Millon-Lugg procedures (*cf.*
above) on both the hydrolysates.

Thyroxine and diiodotyrosine are then calculated as follows:

$E = 0.713 \ ID + 0.286 \ I(1-D)$

where   E is extra Tyrosine in the $SnCl_2$-NaOH hydrolysis.
               I is Total Iodine
            ID is Iodine in Diiodotyrosine
        $I(1-D)$ is Iodine in Thyroxine

PART VII

AROMATIC AMINO ACIDS IN PROTEINS

All values given in the following tables have been calculated to 16.0 per cent of nitrogen. In those instances, where nitrogen figures are not given by the investigators, the amino acid figures have been calculated using the value of N which is given in parenthesis. If the investigators reported the data in amino acid nitrogen as per cent of total nitrogen, then the results have been recalculated to 16.0 per cent of nitrogen but the value of N is omitted from the tables.

Cf. Chapter I, Part VII for comments on "Best Values" and the calculation of the mean with twice the standard error.

Under the heading "Method," the general principle used to estimate tyrosine, tryptophane, and phenylalanine respectively is given. Thus "Millon" refers to one of the adaptations of the Millon test for tyrosine; "Millon-Lugg" refers to Lugg's adaptation of the Millon reaction for both tyrosine and tryptophane; "Folin" indicates the use of phosphomolybdotungstic acid for the estimation of either tyrosine or tryptophane (usually tryptophane); "Kapeller" refers to the Kapeller-Adler method for phenylalanine or one of its modifications, and so forth.

ALBUMINOIDS

Aromatic Amino Acids in *Gelatin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
			per cent	gm.	gm.	gm.	
Jolles-Albanese	Albanese	28			0.0		
Millon, Folin, Kapeller	unpublished		16.6	0.3	0.0	1.9	Pork Skin
Millon, Folin, Kapeller	unpublished		16.0	0.2	0.0	2.3	Bone
Millon, Folin, Kapeller	unpublished		15.4	0.2	0.0	2.1	Coignet
Isolation	Dakin	185	18.0			1.4	
Millon, Folin	Folin	231		trace	0.0		
Millon, Pauly-Hanke	Fürth	248	(16.0)	0.0			
Voisenet	Fürth	249	(16.0)	.	0.0		
Pauly-Hanke	Hanke	280	(16.0)	0.3			
Millon, Folin	Holiday	302	(16.0)	0.0	0.0		
Spectrophotometric	Holiday	302	(16.0)	0.4	0.1		
Rhode-May	Jones	342	(16.0)		0.0		
Kapeller-Adler	Kapeller	350	(16.0)			1.2	
Kollmann	Kollmann	376	(16.0)			0.3*	
Voisenet-Kraus	Kraus	388	(16.0)		0.1		
Rhode-May	May	440	(16.0)		0.0		
Folin	May	440	(16.0)		0.0		
Chromatographic	Gordon	261D				1.9*	
Mean				0.2	0.0	1.8	
* Omitted from mean							

112

ALBUMINOIDS

Aromatic Amino Acids in *Elastins*, *Collagens*, and *Related Proteins*

				Calculated to 16.0 gm. N.		
PROTEIN	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
Elastin	Fischer	Abderhalden 4	(17.1)			3.7
Elastin	Gerngross	Gerngross 256	(17.1)	1.5		
Elastin	Kapeller-Adler	Kapeller 350	(17.1)			3.1
Elastin	Millon-Lugg	Stein 586	17.1	1.5	0.0	
Collagen		Theis 604	(16.0)	1.0		
Lens	Isolation	Hijikata 298	(16.0)	4.5		
Neurogelatin	Millon, Folin, Kapeller	unpublished	14.7	3.5	0.9	4.7
Fish gelatin	Millon, Folin, Kapeller	unpublished	11.8	0.8	0.9	1.9
Fish gelatin	Millon-Lugg	unpublished	11.8	0.8	0.7	

ALBUMINOIDS

Gelatin: There appears to be a slight but significant quantity of tyrosine in gelatin. Gerngross (256) using his specific color test reported that the tyrosine in gelatin varies from 0.0 to 1.0 per cent. The actual quantity present depends on the method used in preparing gelatin from collagen. If strong bleaching agents such as chlorites are used in its preparation, any tryptophane and a large proportion of tyrosine which existed in the original collagen would be destroyed.

Elastin: This protein is somewhat similar in its distribution of the aromatic amino acids to collagen (gelatin).

Neurogelatin: The hot water soluble protein was prepared by extracting brain tissue with slightly acidulated hot water. Neurogelatin is considerably richer in the aromatic amino acids than bone or skin gelatins.

ANIMAL PROTEINS

Aromatic Amino Acids in *Entire Animals*

				Calculated to 16.0 gm. N.			
ANIMAL	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
			per cent	gm.	gm.	gm.	
Rat	Millon, Folin, Kapeller	unpublished	13.3	3.0	0.8	3.5	2 days old
Rat	Millon-Folin	unpublished	13.3	3.0	0.7		2 days old
Rat	Millon, Folin, Kapeller	unpublished	11.1	3.2	1.0	4.3	23 days old
Rat	Millon-Folin	unpublished	11.1	3.6	0.7		23 days old
Rat	Millon, Folin, Kapeller	unpublished	13.4	3.2	0.7	3.8	100 days old
Rat	Millon-Folin,	unpublished	13.4	2.9	0.6		100 days old
Rat	Millon, Folin, Kapeller	unpublished	12.2	3.2	0.8	4.3	18 months old
Rat	Millon-Folin	unpublished	12.2	3.2	0.8		18 months old
Rat	Millon-Folin	unpublished	13.1	3.1	0.6		No protein diet
Chicken	Millon-Folin	Calvery 141	(15.0)	4.6	2.1		Embryo

BLOOD PROTEINS

Aromatic Amino Acids in *Fibrin*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
				per cent	gm.	gm.	gm.	
Cattle	Millon-Lugg, Kapeller	unpublished		13.4	5.0	3.7	6.0	Swift
Cattle	Millon-Lugg, Kapeller	unpublished		9.8	5.5	3.3	8.5	Peptone-Difco
Cattle	Millon, Folin	unpublished		13.3	6.4	3.8		
Cattle	Millon-Lugg	unpublished		13.3	5.9	·3.9		
Cattle	Millon-Lugg	Brand	123	17.7	5.7	3.4		
Cattle	Millon, Folin	Folin	231	(17.0)	5.6	2.8		Witte peptone
Cattle	Millon, Folin	Folin	231	(17.7)	5.9	2.6		
Cattle ?	Millon-Fürth	Fürth	250	(17.7)	4.2			
Cattle ?	Gerngross	Gerngross	256	(17.7)	5.9			
Cattle	Pauly-Hanke	Hanke	280	(17.7)	3.1			
Cattle ?	Rhode-May	Holm	303	(17.7)		4.5		
Cattle ?	Rhode-May	Jones	342	(17.7)		4.0		
Cattle	Millon, Folin	Jorpes	344	16.8	5.1	3.8		
Cattle	Kollmann	Kollmann	376	(17.7)			2.0*	
Cattle ?	Voisenet and Rhode	Komm	377	(17.7)		1.9		
Cattle ?	Voisenet-Kraus	Kraus	389	(16.0)		3.0		Witte peptone
Cattle ?	Millon	Zuwerkalow	699	(16.0)	6.5			Witte peptone
Sheep	Pauly-Hanke	Hanke	280	(17.7)	3.0			
Hog	Pauly-Hanke	Hanke	280	(17.7)	3.1			
"Best Values"					6.0	3.5	₁7	
Mean with 2×S.E.					5.1±0.7	3.4±0.4		
*Omitted from average								

BLOOD PROTEINS

Aromatic Amino Acids in *Hemoglobins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Horse	Fischer	Abderhalden	2	16.8			3.9
Horse	Millon, Kapeller	Block	94	16.7	2.0		6.7*
Horse ?	Millon, Folin	Folin	233	(16.7)	3.0	1.2	
Horse	Millon-Fürth	Fürth	250	(16.7)	2.7		
Horse	Millon, Folin	Jorpes	344	16.8	2.6*	1.1*	
Horse	Kapeller-Adler	Kapeller	350	(16.8)			5.1
Horse ?	Kollmann	Kollmann	376	(16.8)			3.4
Cattle	Millon, Folin, Kapeller	Block	94	16.1	2.0	1.0	6.8
Sheep	Millon, Kapeller	Block	94	16.8	2.1		6.9
Pig	Millon, Folin, Kapeller	unpublished		15.0	2.0	1.1	6.3
Turtle	Millon, Folin, Kapeller	unpublished		15.5	2.6	1.5	7.0
Mean with 2×S.E.					2.4±0.3	1.2	5.8±0.9
* Best Values							

BLOOD PROTEINS

Aromatic Amino Acids in *Globins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Cattle	Millon, Voisenet	Roche	550	16.6	3.1	2.2	
Horse	Millon, Voisenet	Roche	550	16.8	3.2	2.3	
Dog	Millon, Voisenet	Roche	550	16.6	3.4	2.8	
Guinea Pig	Millon, Voisenet	Roche	550	16.7	2.5	1.8	
Human	Millon, Voisenet	Roche	550	16.7	2.7	2.2	
Human	Millon, Folin, Kapeller	unpublished		16.2	3.4	1.4	7.8
Human	Millon-Lugg	unpublished		16.2	3.0	1.3	
Rabbit	Millon, Voisenet	Roche	550	16.6	3.6	2.9	
Sheep	Millon, Voisenet	Roche	550	16.8	3.5	2.5	
Pig	Millon, Voisenet	Roche	550	16.3	3.0	2.2	
Mean with 2 ×S.E.					3.1 ± 0.2	2.2 ± 0.3	

Note contrast between Folin and Millon-Lugg results for tryptophane and those found by the Voisenet formaldehyde method.

BLOOD PROTEINS

Aromatic Amino Acids in *Serum Albumins*

Calculated to 16.0 gm N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
				per cent	gm.	gm.	gm.	
Horse	Millon, Folin	Abderhalden	23	(16.0)	4.2	0.8		$(NH_4)_2SO_4$
Horse	Millon, Folin	Hewitt	296		5.3[a]	0.3[a]		crystalline
Horse	Millon, Folin	Hewitt	296		6.6[b]	1.2[b]		crystalline
Horse	Millon, Folin	Holiday	302	(16.0)	5.9	0.8		
Horse	Spectropho-tometric	Holiday	302	(16.0)	7.3	0.7		
Horse	Millon, Folin	Jorpes	344	(16.0)	4.3	1.0		crystalline
Horse ?	Voisenet and Rhode	Komm	377	(16.0)		2.7		crystalline
Horse ?	Folin	Reiner	543		4.4			
Horse ?	Millon, Folin	Folin	233	(16.0)	4.7	0.5		crystalline
Horse ?	Voisenet	Fürth	249	(16.0)		1.2		
Cattle	Millon, Folin	Abderhalden	23	(16.0)	4.4	0.8		$(NH_4)_2SO_4$
Cattle	Millon, Folin	v. Deseo	193	(16.0)	4.9-5.3	1.0-1.4		$(NH_4)_2SO_4$
Cattle	Millon, Folin	v. Deseo	193	(16.0)	4.4-5.7	0.8-2.0		calf
Cattle	Folin	Reiner	543		4.0			
Human	Millon, Folin	Bálint	46	(16.0)	4.8 ± 0.3	0.7 ± 0.1		Na_2SO_4
Human	Millon, Folin, Kapeller	unpublished		15.7	4.5	0.5	7.9	K_1HPO_4
Human	Millon, Folin	unpublished		14.7	4.4	0.6		$(NH_4)_2SO_4$
Human	Folin	Reiner	543		4.8			
Human	Millon, Folin	Murrill	469	13.5	4.9	0.8		Na_2SO_4
Rabbit	Millon, Folin	Abderhalden	23	(16.0)	5.9	0.5		$(NH_4)_2SO.$
Rabbit	Folin	Reiner	543		5.7			
Monkey	Folin	Reiner	543		3.9			
Dog	Folin	Reiner	543		5.1			
Cat	Folin	Reiner	543		5.1			
Pig	Folin	Reiner	543		3.9			
Sheep	Folin	Reiner	543		5.0			
Rat	Folin	Reiner	543		5.0			
Guinea Pig	Folin	Reiner	543		4.2			
Chicken	Folin	Reiner	543		4.7			
Mean					4.9	1.0		

[a] least soluble crystalline albumin
[b] most soluble crystalline albumin

BLOOD PROTEINS

Aromatic Amino Acids in Serum Globulins

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ANALINE	
				per cent	gm.	gm.	gm.	
Horse	Fischer	Abderhalden	5	(16.0)			3.8	
Horse	Millon, Folin	Abderhalden	23	(16.0)	5.7	1.9		euglob.
Horse	Millon, Folin	Abderhalden	23	(16.0)	5.9	1.7		pseudoglob.
Horse	Millon, Folin	Calvery	144	16.0	5.5	2.0		Pneumococcus antiserum
Horse	Millon, Folin	Calvery	145	(16.0)	5.5	2.1		Pneumococcus antiserum
Horse ?	Voisenet	Fürth	249	(16.0)		3.1		
Horse ?	Millon-Fürth	Fürth	250	(16.0)	3.8			
Horse	Millon, Folin	Holiday	302	(16.0)	7.1	2.2		euglob.
Horse	Spectrophoto-metric	Holiday	302	(16.0)	6.9	2.6		euglob.
Horse	Millon, Folin	Holiday	302	(16.0)	6.3	2.4		pseudoglob.
Horse	Spectrophoto-metric	Holiday	302	(16.0)	5.8	2.9		pseudoglob.
Horse	Millon, Folin	Jorpes	344	15.5	5.5	2.4		
Horse ?	Voisenet and Rhode	Komm	377	(16.0)		2.5		
Horse	Folin	Reiner	543		4.1			
Cattle	Millon, Folin	Abderhalden	23	(16.0)	6.6	1.9		euglob.
Cattle	Millon, Folin	Abderhalden	23	(16.0)	6.6	1.7		pseudoglob.
Cattle	Millon, Folin	v. Deseő	193	(16.0)	4.7−5.9	1.6−2.0		(NH₄)₂SO₄
Cattle	Millon, Folin	v. Deseő	193	(16.0)	3.6−5.1	0.9−2.6		calf
Cattle	Millon, Folin	Folin	231	(16.0)	6.7	2.3		
Cattle	Folin	Reiner	543		4.6			
Human	Millon, Folin	Bálint	46	(16.0)	6.2±0.4	2.0±0.3		Na₂SO₄
Human	Millon, Kapellar	unpublished		14.2	5.6		6.8	K₂HPO₄
Human	Millon, Kapellar	unpublished		14.1	5.1		5.0	(NH₄)₂SO₄
Human	Millon, Folin	Murrill	469	14.0	6.4	2.4		Na₂SO₄
Rabbit	Millon, Folin	Abderhalden	23	(16.0)	5.9	1.6		euglob.
Rabbit	Millon, Folin	Abderhalden	23	(16.0)	5.8	1.6		pseudoglob.
Rabbit	Folin	Reiner	543		4.3			
Monkey	Folin	Reiner	543		5.1			
Cat	Folin	Reiner	543		5.1			
Pig	Folin	Reiner	543		4.0			
Sheep	Folin	Reiner	543		5.8			
Rat	Folin	Reiner	543		4.2			
Guinea Pig	Folin	Reiner	543		5.2			
Chicken	Folin	Reiner	543		4.7			
Mean					5.5	2.1		

BLOOD PROTEINS

Aromatic Amino Acids in *Serum Proteins*

Calculated to 16.0 gm. N.

	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
				per cent	gm.	gm.	gm.	
Human	Millon, Folin	Bálint	46	(16.0)	5.5±0.3	1.4±0.3		
Human	Millon, Folin, Kapeller	Block	105	14.9	4.4		5.4	
Human	Millon, Folin	Block	110	15.4	4.6	1.3		
Human	Millon, Folin	Block	110	15.2	5.0	1.4		Arthritis
Dog	Millon, Folin	Murrill	470	12.2	5.5	2.1		Normal diet
Dog	Millon, Folin	Murrill	470	11.6	6.1	1.8		Reserve Protein
Dog	Millon, Folin	Murrill	470	14.4	5.5	1.7		Regenerated Protein
Dog	Millon, Folin	Murrill	470	12.7	5.9	1.9		Casein diet
Dog	Millon, Folin	Murrill	470	12.2	5.9	1.9		Albumin diet
Dog	Millon, Folin	Murrill	470	14.1	5.8	1.7		Serum diet
Dog	Millon, Folin	Murrill	470	14.2	5.4	1.8		Yeast diet
Cattle	Millon, Folin	v. Deseö	192	(16.0)	4.7	1.8		
Mean with 2×S.E.					5.4±0.3	1.7±0.1		

BLOOD PROTEINS

Aromatic Amino Acids in *Human Pathological Serum and Urine Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
				per cent	gm.	gm.	gm.	
Serum	Millon, Kapeller	unpublished		14.1	5.0		6.0	Myeloma
Serum	Millon, Folin	Murrill	469	14.3	5.6	1.7		Nephritic
Urine	Isolation, Fischer	Abderhalden	11	(16.0)	1.7		1.5	Bence-Jones
Urine	Millon, Folin	Devine	195	14.7	9.6	1.4		Bence-Jones
Urine	Spectrophotometric	Devine	195	14.7	9.2	2.5		Bence-Jones
Urine	Millon, Folin	Calvery	143	18.1	6.8	2.5		Bence-Jones
Urine	Millon, Folin	Folin	231	(16.0)	7.4	1.7		Bence-Jones
Urine	Isolation	Hopkins	308	16.2	4.2	0.8		Bence-Jones
Urine	Millon, Folin	Murrill	469	15.1	5.2	0.9		Nephritic

BLOOD PROTEINS

Aromatic Amino Acids in *Stroma and Cell Proteins*

							Calculated to 16.0 gm. N.
SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
				per cent	gm.	gm.	gm.	
Horse	Millon, Folin	Beach	55	12.9	3.6	1.4		
Horse	Millon, Folin	Jorpes	344	14.8	3.7	1.7		
Cattle	Millon, Folin	Beach	55	13.8	3.4	1.4		
Cattle	Millon, Folin	Erickson	213	13.7	3.6	1.4		embryo
Sheep	Millon, Folin	Beach	55	14.0	3.3	1.3		
Hog	Millon, Folin	Beach	55	13.1	3.4	1.5		
Human	Millon, Folin	Beach	55	13.0	3.3	1.5		
Human	Millon, Folin	Erickson	213	13.1	3.6	1.5		Polycythemia
Mean with 2×S.E.					3.5±0.1	1.5±0.1		
Human	Folin, Kapeller	Block	105	16.1		1.2	8.5	cells

BLOOD PROTEINS

Fibrin: Blood fibrin appears to be the best source of tryptophane readily available, although certain relatively rare purified enzymes and viruses may yield higher amounts.

Hanke's results on tyrosine are of comparative interest only as they appear to be uniformly low.

Hemoglobins and *Globins:* Although the aromatic amino acid composition of globins from various mammals may vary slightly (*cf.* the basic amino acids, Chapter I), the general aromatic amino acid composition of mammalian globins is similar.

Serum Albumins: Sørensen was the first to demonstrate clearly that the composition of a serum albumin preparation, even though crystalline, was a function of its mode of preparation. Hewitt (296) prepared two crystalline albumins from crystalline serum albumin which differed in the tyrosine and especially in tryptophane content. Serum albumins appear to contain approximately 5 per cent of tyrosine, 1 per cent of tryptophane and 6 to 8 per cent of phenylalanine.

Serum Globulins: These substances are not crystallized and appear to be even more heterogeneous than serum albumins. However, serum globulins, as a group, contain somewhat more tyrosine and approximately twice as much tryptophane as serumalbumins.

Proteins in Pathological Conditions: The so called Bence-Jones protein does not appear to be a definite entity, but a group of proteins having the same gross physical properties which differ in chemical composition and in immunological properties.

Stroma Proteins: The yields of tyrosine and tryptophane on hy-

drolysis of 5 mammalian stroma proteins are approximately the same. Little or no species specificity is shown.

BRAIN PROTEINS

Aromatic Amino Acids from *Human Brain Proteins* (*cf.* 89, and unpublished results)

Calculated to 16.0 gm. N.

CAUSE OF DEATH	METHOD	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
		per cent	gm.	gm.	gm.	
Infection	Millon, Folin	13.4	4.7	1.6		
Infection	Millon, Folin, Kapeller	14.1	4.1	0.7	6.8	
Infection	Millon, Folin	14.5	4.2	1.2		male
Infection	Millon, Folin	14.3	4.1	1.3		male
Infection	Millon, Folin	14.1	4.1	1.3		male
Hemorrhage	Millon, Folin, Kapeller	13.7	3.7	1.4	5.0	male
Hemorrhage	Millon, Folin, Kapeller	13.4	4.7	1.4	5.3	male
Hemorrhage	Millon, Folin, Kapeller	14.9	3.8	1.2	4.6	male
Hemorrhage	Mollin, Folin, Kapeller	15.1	4.0	1.2	4.8	male
Suicide	Mollin, Folin, Kapeller	15.1	3.8	1.2	5.2	male
Suicide	Millon, Folin, Kapeller	12.8	4.0	1.4	5.8	male
Suicide	Millon, Folin, Kapeller	14.0	3.8	1.5	4.8	male
Suicide	Millon, Folin, Kapeller	13.2	4.2	1.5	5.0	male
Alcoholism	Millon, Folin, Kapeller	14.9	3.9	1.4	5.6	male
Arteriosclerosis	Millon, Folin, Kapeller	13.0	4.2	1.5	5.0	male
Trauma	Millon, Folin	13.0	4.1	1.5		
Amaurotic Idiocy	Millon, Folin, Kapeller	14.1	5.1	1.2	6.4	
Amaurotic Idiocy	Millon-Lugg	14.1		1.1		
Hemorrhage	Millon, Folin, Kapeller	14.2	3.7	1.2	4.0	female
Diabetes	Mollin, Folin, Kapeller	13.4	4.0	1.3	5.0	female
Infection	Millon, Folin, Kapeller	13.9	4.2	1.3	5.3	female
Alcoholism	Millon, Folin, Kapeller	15.0	3.9	1.3	4.8	female
Arteriosclerosis	Millon, Folin, Kapeller	12.7	4.5	1.3	5.7	female
Arteriosclerosis	Millon, Folin, Kapeller	13.6	4.2	1.4	5.5	female
Unknown	Millon, Rhode, Kapeller	15.6	4.3*	1.5*	4.1*	
Mean with 2×S.E.		14.0	4.1±0.1	1.3±0.1	5.2±0.3	
* Kaplansky (354)						

BRAIN PROTEINS

Aromatic Amino Acids in *Animal* Brain Proteins (cf. 89, 105, 354, and unpublished).

Calculated to 16.0 gm. N.

ANIMAL	METHOD	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
		per cent	gm.	gm.	gm.	
Monkey	Millon, Folin, Kapeller	13.4	4.7	1.6		
Monkey, male	Millon, Folin	14.3	3.8	1.1	5.1	
Monkey, male	Millon, Folin, Kapeller	14.4	4.1	1.1	4.3	
Monkey, male	Millon, Folin, Kapeller	14.9	3.9	1.1	4.1	
Monkey, male	Millon, Folin, Kapeller	13.9	4.0	1.4	5.4	
Monkey, male	Millon, Folin, Kapeller	14.6	4.2	1.2	4.0	
Monkey, female	Millon, Folin, Kapeller	14.0	3.9	1.1	4.7	
Monkey, female	Millon, Folin, Kapeller	14.8	3.5	1.2	4.3	
Monkey, female	Millon, Folin	14.2	4.0	1.1		
Monkey, female	Millon, Folin	14.5	3.7	1.0		
Monkey, female	Millon, Folin	14.6	3.9	1.2		
Monkey, female	Millon, Folin, Kapeller	15.1	4.1	1.1	3.8	
Monkey, female	Millon, Folin, Kapeller	14.2	3.7	1.3	4.4	
Monkey, female	Millon, Folin, Kapeller	14.6	4.3	1.3	4.6	
Sheep, male	Millon, Folin, Kapeller	14.4	4.1	1.1	5.1	
Sheep, male	Millon, Folin, Kapeller	11.9	3.8	0.9	4.2	
Sheep, male	Millon, Folin, Kapeller	14.7	3.9	1.2	4.6	
Sheep, male	Kapeller	13.2			5.0	
Sheep	Millon, Folin	12.5	4.6	1.4		
Sheep, male	Millon, Folin, Kapeller	13.5	4.2	1.2	4.5	
Sheep, male	Millon, Folin, Kapeller	14.4	3.0	0.7	4.4	
Sheep, male	Millon, Folin, Kapeller	14.6	4.3	1.1	4.5	
Sheep, female	Millon, Folin, Kapeller	14.4	4.0	1.2	4.6	
Sheep, female	Millon, Folin, Kapeller	14.7	4.1	1.1	4.5	
Sheep, female	Millon, Folin	15.3	3.4	0.7		
Sheep, female	Millon, Folin, Kapeller	13.0	4.1	1.2	5.0	
Sheep, female	Millon, Folin, Kapeller	15.0	4.0	1.1	5.1	
Sheep	Millon, Rhode, Kapeller	15.2	4.6*	1.6*	4.1*	
Rat	Millon, Folin, Kapeller	14.3	4.0	1.0	3.7	1 day old
Rat	Millon, Folin, Kapeller	14.5	4.0	1.2	4.0	6 day old
Rat	Millon, Folin, Kapeller	14.4	3.6	1.2	4.5	22 day old
Rat	Millon, Folin, Kapeller	14.7	3.8	1.2	5.3	adult
Rat	Millon, Folin, Kapeller	15.0	3.5	1.1	4.6	adult
Rat	Kapeller	15.2			4.2*	
Cattle	Millon, Folin, Kapeller	14.3	4.7	1.2	5.4	
Cattle	Millon, Folin	13.0	4.7	1.2		
Cattle	Millon, Folin	14.7	4.8	1.2		
Cattle	Millon, Rhode, Kapeller	15.2	4.6*	1.6*	4.1*.	
Pig	Millon, Folin, Kapeller	15.3	3.7	1.3	4.9	
Pig	Millon, Folin, Kapeller	15.4	3.7	1.1	5.4	
Rabbit	Millon, Rhode, Kapeller	15.1	4.8	1.6	4.1	
Rabbit	Millon, Folin	12.9	4.1	1.2		
Rabbit	Millon, Folin	12.6	4.2	1.3		
Guinea Pig	Millon, Folin	13.8	4.8	1.2		
Cat	Millon, Rhode, Kapeller	(15.2)	4.3*	1.5*	4.1*	
Dog	Millon, Rhode, Kapeller	15.1	4.7*	1.6*	4.0*	
Mouse	Millon, Rhode	14.9	4.8*	1.5*		
Chicken	Millon, Rhode	15.3	4.6*	1.5*		
Frog	Millon, Rhode, Kapeller	15.3	4.5*	1.5*	4.2*	
Fish	Millon, Rhode	14.9	4.7*	1.6*		
Mean with 2×S.E.			4.1±0.1	1.2±0.1	4.5±0.2	
* Kaplansky (354)						

BRAIN PROTEINS

There appears to be little if any difference in the aromatic amino acids in the proteins of the entire mammalian, reptilian, or piscine brain.

The slightly higher values for tryptophane and the lower figures for phenylalanine found by Kaplansky are presumably due to the methods employed in the analyses. Kaplansky used the original Kapeller-Adler procedure for phenylalanine.

EGG PROTEINS

Aromatic Amino Acids in *Crystalline Egg Albumin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
			per cent	gm.	gm.	gm.	
Kapeller	Arnow	37	15.1			5.6	11 deter.
Millon, Folin, Kapeller	Bernhart	73	15.3	4.1	1.2	5.6	
Millon-Lugg, Kapeller	unpublished		13.9	4.0	1.5	7.9	not crystalline
Millon-Lugg	Brand	123	(14.9)	4.3	1.3		
Millon, Folin	Calvery	139	(15.4)	4.4	1.3		
Millon-Lugg	Chibnall	160	15.8	4.2	1.3		
Millon, Folin	Folin	231	(15.4)	4.4	1.3		
Millon, Folin	Folin	231	(15.4)	4.2	1.4		
Millon, Folin	Folin	233	(15.4)	4.2	1.2		
Millon, Folin	Folin	233	(15.4)	4.1	1.2		
Voisenet	Fürth	249	(15.4)		1.9		
Millon-Fürth	Fürth	250	(15.4)	3.9			
Pauly-Hanke	Hanke	280	(15.4)	2.4*			
Millon-Folin	Hanke	281	(15.4)	3.7			
Rhode-May	Jones	342	(15.4)		2.4		
Voisenet and Rhode	Komm	377	(15.4)		1.5		
Rhode-May	May	440	(15.4)		1.2		
Folin	May	440	(15.4)		1.3		
Isolation, Fischer	Osborne	497	15.5	1.8*		5.3	
Folin	Pottinger	527	(15.4)		1.3		
Millon	Reiter	544	(15.4)	3.9			
Rhode-May	Tomiyama	614	(15.4)		1.3		
Millon	Zuwerkalow	699	(15.4)	4.6			
Mean with 2 ×S.E.				4.2±0.1	1.4±0.2	6	
* Omitted from mean							

EGG PROTEINS

Aromatic Amino Acids in *Egg Proteins* other than Albumin

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Conalbumin	Rhode-May	Jones	342	(16.0)		5.0	
Vitellin	Isolation, Fischer	Abderhalden	13	(15.0)	1.7		3.0
Vitellin	Millon, Folin	Calvery	140	15.0	5.3*	1.3	
Vitellin	Rhode-May	Jones	342	(15.0)		2.6	
Vitellin	Voisenet and Rhode	Komm	377	(15.0)		1.5*	
Vitellin	Rhode-May	May	440	(15.0)		1.9	
Vitellin	Isolation, Fischer	Osborne	495	16.3	3.3		2.5
Livetin	Millon, Folin	Jukes	348	15.5	6.3	1.3	
Livetin	Voisenet and Rhode	Komm	377	(15.0)		1.8	
Egg White	Millon, Folin	Calvery	141	(15.0)	4.6	1.7*	
Egg White	Millon, Folin	Folin	233	(15.0)	4.3	1.4	
Egg White	Millon, Voisenet	McFarlane	448	15.2	5.4*	1.5	
Egg White	Folin	McFarlane	448	15.2		1.4	
Egg White	Rhode-May	McFarlane	448	15.2		1.7	
Egg White	Kapeller	Virtanen	663	12.1			5.5*
Yolk	Millon-Lugg, Kapeller	unpublished		14.6	4.4	1.5	5.7
Yolk	Millon, Folin	Calvery	141	(15.0)	5.8	1.7*	
Yolk	Voisenet and Rhode	Komm	377	(15.0)		1.3	
Yolk	Millon, Voisenet	McFarlane	448	14.3	5.6*	1.7	
Whole Egg	Millon-Lugg, Kapeller	unpublished		14.1	4.2	1.5*	5.9
Whole Egg	Millon, Folin	Patton	512	16.0	4.3*	1.1	
Ovomucoid	Fischer	Abderhalden	5	(13.5)			5
Ovomucoid	Millon, Voisenet	McFarlane	448	13.5	4.2	2.2	

* Best Values

EGG PROTEINS

Egg Albumin: The high value for tryptophane reported by Jones was based on casein with 2.4 per cent of tryptophane as the color standard. If the casein had been taken as containing 1.4 per cent tryptophane, then Jones' value would have checked the rest.

FOODS

Aromatic Amino Acids in *Feeds and Foods*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
			per cent	gm.	gm.	gm.	
Bread	Millon, Folin, Kapeller	unpublished	11.2	4.6	1.5	5.3	
Bread	Millon, Folin, Kapeller	unpublished	11.3	4.2	1.0	4.8	
Flour	Millon, Folin, Kapeller	unpublished	12.8	3.8	0.8	5.5	5 preps.
Cereal	Millon, Folin, Kapeller	unpublished		3.0	1.5	5.6	Wheatena
Cereal	Millon-Lugg	unpublished		3.6	1.0		Wheatena
Cereal	Millon, Folin, Kapeller	unpublished	12.3	3.5	1.2	5.5	Ralston
Cereal	Millon-Lugg	unpublished	12.3	3.5	0.8		Ralston
Cereal	Millon, Folin, Kapeller	unpublished		3.5	2.4	4.1	Cream Farina
Cereal	Millon, Lugg	unpublished		3.4	1.0		Cream Farina
Cereal	Millon, Folin, Kapeller	unpublished	13.6	3.6	2.0	5.6	Cream of Wheat
Cereal	Millon-Lugg	unpublished	13.6	3.5	0.8		Cream of Wheat
Cereal	Millon, Kapeller	unpublished		4.5		4.5	New Cream of Wheat
Cereal	Millon-Lugg	unpublished		4.0	1.4		New Cream of Wheat
Cereal	Millon, Folin, Kapeller	unpublished		2.1	1.0	4.6	Puffed Sparkies
Cereal	Millon-Lugg	unpublished		1.9	0.5		Puffed Sparkies
Cereal	Millon-Lugg, Kapeller	unpublished		2.3	0.6	2.8	Cerevim
Soybean Meal?		Heinrich 286	?	5.6		3.8	
Lupine Meal?		Heinrich 286	?	5.6		4.5	
Meat Scraps	Millon, Folin, Kapeller	unpublished		3.2	0.6	4.5	
Meat Scraps	Millon-Lugg	unpublished			0.8		
Meat Meal	Millon, Folin	McFarlane 447		2.9	1.3		
Meat Meal	Pauly, Voisenet	McFarlane 447		2.4	0.9		
Meat Meal	Pepsin-Trypsin, Voisenet	McFarlane 447		3.0	1.0		
Meat Meal	Trypsin, Voisenet	McFarlane 447			1.8		
Tankage	Millon, Folin, Kapeller	unpublished		2.7	0.8	6.0	
Tankage	Millon, Folin	McFarlane 447		3.0	0.7		
Tankage	Pauly, Voisenet	McFarlane 447		2.7	0.6		
Fish Meal	Millon, Folin, Kapeller	unpublished	11.6	3.3	0.8	4.8	Menhaden
Fish Meal	Millon, Folin	McFarlane 447		2.6	1.3		
Fish Meal	Pauly, Voisenet	McFarlane 447		2.2	1.2		
Fish Meal	Pepsin-Trypsin, Millon, Folin	McFarlane 447		2.9	1.4		
Fish Meal	Pepsin-Trypsin, Voisenet	McFarlane 447			1.6		
Fish Meal	Millon, Folin	Pottinger 526		3.3	0.8		Haddock
Codliver Meal	Folin	McFarlane 447			2.0		
Codliver Meal	Pauly, Voisenet	McFarlane 447		3.6	0.8		
Fish Gelatin	Millon, Folin, Kapeller	unpublished	11.8	0.8	0.9	1.9	Stick
Fish Gelatin	Millon-Lugg	unpublished	11.8	0.8	0.7		Stick
Butter-milk	Millon, Folin	McFarlane 447		5.5	1.8		
Butter-milk	Pauly, Voisenet	McFarlane 447		5.3	1.5		
Butter-milk	Pepsin-Trypsin, Millon, Folin	McFarlane 447		6.4	1.3		
Butter-milk	Trypsin, Voisenet	McFarlane 447			1.7		
Butter-milk	Pepsin-Trypsin, Voisenet	McFarlane 447			3.0		

FEEDS AND FOODS

Bread and Flour: The nutritional superiority of bread made with 6 per cent milk solids and a good grade of yeast is shown in the tryptophane content which is significantly higher than that found in five widely used white flours and farinas.

Cereals: It appears that the use of whole wheat cereals and especially those with added wheat germ are superior in tryptophane content to those which have been subjected to excessive heat and pressure and subsequent rapid release of pressure. The non-specificity of the Folin phosphomolybdotungstic acid tryptophane method is demonstrated in this group of analyses. The Lugg adaptation of the Millon method is preferred in the analysis of protein preparations containing a large quantity of carbohydrate.

Meat and Fish Residues: These protein concentrates contain less tryptophane than is generally recognized. Their chief function in animal nutrition appears therefore to be to supply lysine (*cf.* Chapter I) rather than tryptophane.

Milk Solids: Dried milk is an excellent commercial source of tryptophane.

ANIMAL HORMONES AND ENZYMES

Aromatic Amino Acids in *Hormones and Nonmetallic-Enzymes*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	
				per cent	gm.	gm.	
Insulin	Millon-Lung	Chibnall	160	15.5	12.9*		
Insulin	Kapeller	unpublished		15.5		◄	8.4ᵃ
Insulin	Spectrophotometric	Holiday	302	(15.5)	13.1	0.0	
Insulin	Millon-Folin	Jensen	320	15.5	12		
Insulin	Millon-Folin	duVigneaud	660	(15.7)	12.4		
Pepsin	Millon, Folin	Calvery	146	15.4	10.8	2.3	
Pepsin	Millon, Folin	Calvery	146	15.2	8.7	2.2	heat coagulum
Pepsin	Millon, Folin	Calvery	146	15.4	12.0	2.3	heat filtrate
Thyroglobulin-Human	Millon, Folin	Cavett	152	(16.0)	3.3	2.0	0.3ᵇ, 0.3ᶜ
Thyroglobulin-Colloid	Millon, Folin	Cavett	152	(16.0)	3.4	2.2	0.05ᵇ, 0.0ᶜ
Thyroglobulin-Adenoma	Millon, Folin	Cavett	152	(16.0)	3.2	2.1	0.3ᵇ, 0.1ᶜ
Thyroglobulin-Exophthalmic	Millon, Folin	Cavett	152	(16.0)	3.1	2.1	0.5ᵇ, 0.2ᶜ
Thyroglobulin-Swine	Millon-Lugg	Brand	122	15.8	3.0	1.9	0.7ᵇ, 0.3ᶜ
Thyroglobulin-Pig	Millon, Folin	Eckstein	202		5.5	2.2	
Chymotrypsinogen	Millon-Lugg	Brand	125	16.2	2.9	5.4	
Trypsin	Millon-Lugg	Brand	127	(16.0)		4.6	
Trypsinogen	Millon-Lugg	Brand	127	(16.0)		2.9	
Pituitary Lactogenic	Millon, Hopkins	Li	419	(16.0)	5.7	2.5	Beef
Pituitary Lactogenic	Millon-Lugg	Li	419	(16.0)		1.3	Beef
Pituitary Lactogenic	Millon	Li	419	(16.0)	4.5		Sheep
Pituitary Pressor	Millon-Arnow	Potts	528	(16.0)	11.9		
Pituitary Oxytocic	Millon-Arnow	Potts	528	(16.0)	14.2		
Gonadotropin		Evans	214		7.0	1.5	
Secretin	Millon, Lugg	Ågren	25	14.4	0	0	

ᵃ Phenylalanine
ᵇ Diiodotyrosine
ᶜ Thyroxine
* Best Values.

ANIMAL HORMONES AND ENZYMES

Insulin: The high content of tyrosine and the lack of tryptophane is striking.

Pepsin: This protein, too, is rich in tyrosine, but also contains a relatively high amount of tryptophane. The differences in amino acid composition with the mode of preparation of the protein for analysis should be noted. (Heat coagulation.)

Thyroglobulin: The presence of thyroxine and diiodotyrosine characterize this hormone.

Chymotrypsinogen: This enzyme is one of the few proteins with an inversed tyrosine to tryptophane ratio. Its yield of tryptophane is unusually high.

Pressor and Oxytocic Hormones: These proteins, like pepsin and insulin, contain an uncommonly large quantity of tyrosine.

KERATINS

Aromatic Amino Acids in *Eukeratins*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Human Hair	*Millon, Folin	Block	109	16.6	2.9	1.3*	
Human Hair	Millon, Kapeller	Block	97	15.4	3.1*		2.7
Lamb Wool	Isolation	Abderhalden	16		2.8		
Lamb Wool	Millon, Kapeller	Block	97	15.4	4.7		4.2*
Lamb Wool	Millon, Folin,	Folin	231	(15.4)	5.9*	1.5*	
Lamb Wool	Gerngross	Gerngross	256		5		
Lamb Wool	Millon-Arnow	Gordon	261	(15.4)	4.3		
Lamb Wool	Isolation-Martin	Martin	439				3.6
Lamb Wool		Rutherford	560	(16.8)	5.5		
Lamb Wool	Chromatographic	Gordon	261x		5.8		1.5
Camel Hair	Millon, Folin, Kapeller	Block	97	15.1	3.3	0.8	4.3
Chimpanzee Hair	Millon, Folin,	Block	109	16.7	3.2	1.4*	
Hog Hair	Millon, Folin, Kapeller	unpublished		15.1	3.6	1.1	3.3
Goat Hair	Millon, Folin, Kapeller	Block	97	16.2	3.0	0.9	4.5
Cattle Hair	Millon, Folin	Block	109	15.3	3.4*	1.4*	
Cattle Hair	Gerngross	Gerngross	256		4		
Horse Hair	Isolation	Abderhalden	9	(15.0)	3.4		
Cattle Horn	Isolation, Fischer	Abderhalden	16		3.8		2.0
Cattle Horn	Millon, Folin, Kapeller	Block	97	16.1	3.7		4.0
Cattle Horn	Millon, Folin,	Folin	231	(16.0)	5.3*	1.4*	
Rhinoceros Horn	Millon, Folin, Kapeller	Block	97	15.6	8.8	1.7	5.1
Pig Hoof	Millon, Folin, Kapeller	unpublished		14.8	6.5	1.3	3.7
Emu Bill	Millon, Folin, Kapeller	unpublished		13.2	10.7	1.0	3.4
Black Goose Bill	Millon, Folin, Kapeller	unpublished		10.6	13.0	1.9	7.2
Iguana Bill	Millon, Folin, Kapeller	unpublished		15.5	7.0	0.8	4.9
Goose Feathers	Isolation	Abderhalden	10	(15.0)	3.8		
Hen Feathers	Millon, Folin, Kapeller	Block	97	15.5	2.3	0.7	5.5
? Feathers	Millon-Fürth	Fürth	250	(15.5)	3.3*		
Porcupine Quills	Millon, Folin, Kapeller	Block	97	15.8	3.3	0.9	3.6
Echidna Quills	Millon, Folin, Kapeller	Block	97	15.2	9.6	2.3	7.2
Egg Shell-Membrane	Millon, Folin,	Calvery	141	(16.6)	4.5*	2.7*	
Egg Shell-Membrane	Millon, Folin,	Calvery	142	16.6	2.5	2.5	

* Best Values.

KERATINS

Aromatic Amino Acids in *Skin and Neurokeratins*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Human-Skin	Millon, Folin	Eckstein	203	14.2	3.9	2.0	
Human-Skin	Millon, Folin	Wilkerson	678	15.1	6.0	1.6	
Snake-Skin	Millon, Folin, Kapeller	unpublished		15.2	5.5	1.0	4.1
Turtle-Scutes	Millon, Folin, Kapeller	Block	96	14.1	14.9	2.6	5.9
Pelican-Excresence	Millon, Folin, Kapeller	Block	96	14.0	6.5	1.0	4.9
Whale-Baleen	Millon, Folin, Kapeller	Block	96	14.1	5.7	1.1	3.2
Neurokeratin	Isolation	Argiris	32	14.2	5.2		
Neurokeratin	Millon, Folin, Kapeller	Block	96	13.3	4.6	1.3	5.2
Neurokeratin-trypsin	Millon, Folin Kapeller	unpublished		11.1	5.1	1.8	7.2
Neurokeratin-pepsin	Millon, Folin, Kapeller	unpublished		10.0	3.7	1.3	7.2
Neurokeratin-papain	Millon, Folin, Kapeller	unpublished		10.9	5.7	1.5	4.8

KERATINS

Aromatic Amino Acids in *Egg Casings, Gorgonia, Silk Fibroin, etc.*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Egg Casing-Scyllium	Isolation, Fischer	Pregl	529	15.1	11.2		3.5
Egg Casing-Salmon	Millon, Folin	Young	694	15.3	5.3	1.5	
Gorgonia	Millon, Rhode, Kapeller	Block	96	14.1	14.8	?	6.5
Plexaurella	Millon, Rhode, Kapeller	Block	96	13.7	15.8	?	7.6
Spongin	Millon, Rhode, Kapeller	Block	96	13.0	1.0	?	4.1
Silk Fibroin	Isolation, Fischer	Abderhalden	20	19.0	9.3		1.3
Silk Fibroin	Isolation plus spectro-graphic	Bergmann	69	19.0	11.1		
Silk Fibroin	Millon, Pauly	Fürth	248	(19.0)	9		

KERATINS

Eukeratins: In contrast to the remarkable uniformity in the molecular ratios of the diamino acids in eukeratins (*cf.* Chapter I), their yield of aromatic amino acids varies widely. Thus figures as low as 3 per cent and as high as 13 per cent of tyrosine in different eukeratins have been reported by the same investigators.

Skin and Neurokeratins: Neurokeratins have been classified with skin because of their keratin properties and apparent embryological origin from the ectoderm. These proteins are relatively, but not extraordinarily, rich in the aromatic amino acids.

Gorgonin: Gorgonins from *Gorgonia flabellum* and from *Plexaurella dichotoma* yield relatively large quantities of tyrosine and phenylalanine but appear to be devoid of tryptophane. If sole reliance had been placed on the Folin phosphotungstomolybdic acid reagent then 6.1 per cent of tryptophane would have been

reported in gorgonin from *G. flabellum* and 5.4 per cent of tryptophane in *P. dichotoma.*

Sponges: Spongin, a protein which many 'zoological text books still classify as being chemically similar to silk fibroin, is unique among keratins in containing little tyrosine and apparently no tryptophane.

LIVER PROTEINS

Aromatic Amino Acids in *Liver Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Beef	Millon, Folin, Kapeller	unpublished		13.3	3.6	1.2	7.3
Beef	Millon-Lugg, Kapeller	Beach	59		4.6	1.8	6.1
Rat	Millon, Folin	Lee	411		4.8	1.6	
Cat-Albumin	Millon, Voisenet, Kapeller	Urban	625	15.4	4.4	3.0	4.4
Cat-Globulin	Millon, Voisenet, Kapeller	Urban	625	14.8	3.5	2.9	4.7
Cat-Whole	Millon, Voisenet, Kapeller	Urban	625	15.0	3.7	2.9	4.6
Human	Millon, Kapeller	Block	105	13.6	3.4		7.3
Cod	Millon-Lugg, Kapeller	unpublished			3.8	1.4	5.8
Nucleo protein	Millon-Lugg	Greenstein	266	15.7	$3.9 \pm .2$	$1.5 \pm .2$	
Tumor	Millon-Lugg	Greenstein	267	15.5	3.7	1.5	

LIVER PROTEINS

The large discrepancy between the tryptophane values of Urban and the others reported in the table may be primarily the result of differences in analytical methods.

METALLOPROTEINS

Aromatic Amino Acids in *Metalloproteins* other than Hemoglobin

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	
				per cent	gm.	gm.	
Cytochrome C	Millon-Lugg	Theorell	605	15.4	5.6	1.2	
Hemocyanin	Millon, Voisenet	Roche	550	15.6	4.6	5.7	Mollusc
Hemocyanin	Millon, Voisenet	Roche	550	16.8	4.4	5.4	Crustacea
Hemerytherine	Millon, Voisenet	Roche	550	16.8	5.9	6.0	Siphuncle
Ferritin	Millon, Voisenet, Kapeller	Kuhn	396	8.4	12.2	1.8	1.8% phenyl-alanine

METALLOPROTEINS

The high values reported for tryptophane using a modification of the Voisenet reaction must be accepted with reserve for the time being. The disproportionately large quantity of tyrosine in ferritin as compared to the other aromatic amino acids is noteworthy.

MILK PROTEINS

Aromatic Amino Acids in Casein from Cow's Milk

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Harris	Jolles-Albanese	Albanese	28			1.8	
Sheffield	Jolles-Albanese	Albanese	28			2.4	
deaminated	Jolles-Albanese	Albanese	28			2.5	
acid hydrolyzed	Jolles-Albanese	Albanese	28			0.0*	
	Millon, Folin	Beach	55	15.1	6.6	1.5	
	Millon, Folin	Beach	57	14.5	6.6 ± .1	1.2 ± .1	
Harris	Millon, Folin, Kapeller	unpublished		14.8	5.6	1.3	6.3
Labco	Millon, Kapeller	unpublished		15.8	5.3		4.8
Labco hydrolyzed	Millon-Lugg, Kapeller	unpublished		12.2	5.2	?	5.6
Labco hydrolyzed	Millon-Lugg, Kapeller	unpublished		12.3	6.4	?	6.2
Difco hydrolyzed	Millon-Lugg, Kapeller	unpublished		7.4	0.4*	?	4.3
Difco hydrolyzed	Millon-Lugg, Kapeller	unpublished		10.5	2.1*	?	4.6
	Rhode-May	Csonka	177	(15.4)		2.2	
	Millon, Rhode-May	Csonka	181		6.5	2.1	
	Dakin, Isolation	Dakin	183			1.7	
	Millon, Folin	v. Deseö	192	(15.4)	5.8	1.2	
Hammarsten	Millon, Folin	Folin	231	(15.4)	5.6	1.6	
Hammarsten	Millon, Folin	Folin	232	(15.4)	6.6	1.5	
Cohn	Millon, Folin	Folin	232	(15.4)	6.8	1.5	
	Millon-Fürth	Fürth	250	(15.4)	7.0		
	Pauly-Hanke	Hanke	280	(15.4)	4.7		
	Millon-Folin	Hanke	281	(15.4)	5.6		
	Millon, Folin	Holiday	302	(15.4)	6.2	1.2	
	Spectrophotometric	Holiday	302	(15.4)	7.3	1.0	
	Rhode-May	Holm	303	(15.4)		2.3	
	Bromination	Homer	305	(16.0)		1.3	
	Isolation	Hopkins	307	(16.0)		1.5	
	Rhode-May	Jones	342	(15.4)		2.3	
	Kapeller-Adler	Kapeller	350	(15.4)			5.2
	Kollmann	Kollmann	376	(15.4)			3.2*
	Millon, Folin	Kovács	387	(15.4)	6.2	1.7	
	Voisenet-Kraus	Kraus	388	(15.4)		1.3	
	Millon, Kapeller	Kuhn	393	(15.4)	9.0		5.0
	Millon-Lugg	Lugg	432	15.1	6.9	1.4	
	Rhode-May	May	440	15.4		1.6	
	Folin	May	440	(15.4)		1.6	
	Millon, Folin	McFarlane	447		6.6	1.4	
	Pauly, Voisenet	McFarlane	447		6.6	1.2	
	Voisenet and Rhode	McFarlane	447			2.4	
	Pepsin-trypsin, Folin	McFarlane	447		7.6	1.5	
	Trypsin, Voisenet	McFarlane	447			1.3	
	Pepsin-trypsin, Voisenet	McFarlane	447			1.7	
	Isolation	Onslow	484			1.0–1.5	
English	Onslow	Onslow	485			2.0	
Merck	Onslow	Onslow	485			2.2	
	Isolation, Fischer	Osborne	502	15.6	4.0*		2.4*
	Millon, Folin	Plimmer	521	15.2	6.1	1.5	
	Bromination	Plimmer	522	(15.2)		1.6	
	Millon, Folin	Plimmer	523	14.1	7.3	1.6	
	Folin	Pottinger	527	(15.4)		2.3	
	Isolation	Reach	541		4.6*		
	Voisenet-Rhode	Rhode	545			2	
Fresh	Hopkins-Shaw	Shaw	576	(15.4)		1.4	
B.D.H.	Hopkins-Shaw	Shaw	576	(15.4)		1.1	
Kahlbaum	Hopkins-Shaw	Shaw	576	(15.4)		1.0	
	Rhode-May	Shaw	577	(15.4)		2.5	
	Hopkins-Shaw	Shaw	577	(15.4)		1.4	

MILK PROTEINS (Continued)

Aromatic Amino Acids in *Casein* from Cow's Milk

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
Baryta hydrolysate	Rhode-May	Shaw 577	(15.4)		1.4	
Baryta hydrolysate	Hopkins-Shaw	Shaw 577	(15.4)		1.2	
Casein standard	Rhode-Sullivan	Sullivan 599	(15.4)		2.5	
Tryptophane standard	Rhode-Sullivan Rhode-Thomas	Sullivan 599	(15.4)		1.3	
		Thomas 608	(15.4)		1.8	
		Toennies 613	14.8		1.3	
	Rhode-May	Tomiyama 614	(15.4)		1.8	
	Millon	Zuwerkalow 699	(15.4)	7.1		
Mean with 2×S.E.				6.4±0.4	1.5±0.1[a] 2.0±0.3[b]	5.2±0.5

* Omitted from mean.

[a] Methods other than Voisenet-Rhode (37 values).

[b] Voisenet-Rhode methods only (12 values).

MILK PROTEINS

Aromatic Amino Acids in *Caseins* other than from Cow's Milk

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
Human	Isolation, Fischer	Abderhalden 17	(15.5)	4.8		2.9
Human	Millon, Folin	Beach 55	15.1	6.6	1.5	
Human	Millon, Folin	Plimmer 521	14.4	6.1	1.2	
Human	Bromination	Plimmer 522	14.4		1.1	
Sheep	Millon, Folin	Kovács 387	(15.3)	6.2	1.7	
Goat	Millon, Folin	Kovács 387	(15.3)	5.0	1.5	
Horse	Millon, Folin	Kovács 387	(15.3)	5.6	1.2	
Donkey	Millon, Folin	Kovács 387	(15.3)	4.8	0.9	

MILK PROTEINS

Aromatic Amino Acids in *Lactalbumins* (Whey Proteins)

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
Cow	Jolles-Albanese	Albanese 28			2.8	
Cow	Millon-Lugg, Kapeller	unpublished	13.8	3.9	2.2	5.6
Cow	Millon, Folin	Beach 57	14.2	5.3±0.1	2.0±0.1	
Cow	Rhode-May	Jones 342	15.5		2.8	
Cow	Fischer	Jones 340	15.4			1.3
Cow	Rhode-May	May 440	(15.5)		2.5	
Cow	Millon, Folin	Plimmer 521	14.2	4.1	2.1	
Cow	Bromination	Plimmer 522	(14.2)		2.1	
Human	Millon, Folin	Beach 57	13.7	6.1±0.1	2.0±0.1	
Human	Millon, Folin	Plimmer 521	14.6	4.9	2.7	
Human	Bromination	Plimmer 522	(14.6)		2.7	

MILK PROTEINS

Aromatic Amino Acids in *Milk Proteins* other than Casein and Lactalbumin

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
β-Lactoglobulin	Millon, Folin, Kapeller	Bolling 112	15.5	4.6	1.8	5.3
β-Lactoglobulin	Millon-Lugg	Bolling 112	15.5	4.3	2.0	
β-Lactoglobulin	Millon-Lugg	Brand 128	15.6	3.9	2.0	
Whole Milk	Millon-Lugg, Kapeller	unpublished		5.0		5.3
Whole Milk	Millon, Folin, Kapeller	unpublished	15.2	5.5	1.6	6.1
Whole Human Milk	Millon, Folin, Kapeller	unpublished	15.2	5.1	1.9	5.9
Whole Human Milk	Millon-Lugg	unpublished	15.2		1.9	

MILK PROTEINS

Casein: Although some of the variations in the reported analytical values for the aromatic amino acids in casein are due to changes in the composition of the protein itself, the large differences especially in tryptophane appear to be due to the methods employed. The Voisenet-Rhode aldehyde procedures usually give double the value for tryptophane found by the Folin, Millon-Lugg, or other methods. This fact has led to several rather acrimonious, though relatively fruitless, controversies.

Although the tyrosine content of caseins prepared from the milks of various animals appears to remain approximately constant, the quantities of tryptophane seem to vary significantly.

Other Milk Proteins: The lactalbumins from human and cow's milk contain more tryptophane than do the caseins, consequently human milk protein yields decidedly more tryptophane than the entire protein from cow's milk.

MUSCLE PROTEINS

Aromatic Amino Acids in Animal Muscle Proteins

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	
				per cent	gm.	gm.	
Beef	Rhode-May	Jones	342	(16.0)		1.3	
Beef	Isolation, Fischer	Osborne	498	16.2	2.2		3.2[a]
Beef	Folin	Pottinger	527	(16.0)		0.9	
Beef	Millon, Folin, Kapeller	unpublished		16.1	3.4	1.3	4.9[a]
Beef	Millon, Folin, Kapeller	Beach	59		4.3[b]	1.4[b]	4.9[a]
Beef, Myosin	Millon-Lugg	Bailey	44	16.6	3.2	0.8	
Rat, normal diet	Millon, Voisenet	Roche	548	15.8	3.0	1.8	
Rat, starved	Millon, Voisenet	Roche	548	16.7	2.8	1.6	
Rat, protein starved	Millon, Voisenet	Roche	548	16.3	2.5	1.4	
Rat	Millon, Folin	Lee	411		4.4	1.2	
Rabbit-Myogen	Millon-Lugg	Bailey	44	16.6	4.1	1.5	
Rabbit-Myosin	Millon-Lugg	Bailey	44	16.7	3.2	0.8	} Total Protein
Rabbit-Globulin	Millon, Folin	Folin	233	(16.0)	3.9	1.6	impure
Rabbit-Globulin	Millon, Folin	Folin	233	(16.0)	3.9	1.0	purified
Rabbit-Myosin	Voisenet and Rhode	Komm	377	(16.0)		1.5	
Rabbit-Myosin	Fischer	Sharp	575	16.8			3.8[a]
Rabbit		Kandatu	349	(16.0)	4.3	1.8	1.8[a]
Veal	Millon, Lugg, Kapeller	Beach	59		4.9[b]	1.4[b]	4.4[a]
Lamb	Millon, Lugg, Kapeller	Beach	59		4.9[b]	1.4[b]	4.5[a]
Pork	Millon, Lugg, Kapeller	Beach	59		4.4[b]	1.3[b]	4.0[a]
Dog-Myosin	Millon-Lugg	Bailey	44	16.6	3.2	0.7	
Chicken-Myosin	Millon-Lugg	Bailey	44	16.6	3.2	0.8	
Chicken	Millon, Folin	Gurevich	272	(16.0)	2.4—3.4	0.8—1.5	
Chicken	Isolation, Fischer	Osborne	493	(16.0)	2.2		3.5[a]
Chicken	Rhode-May	Tomiyama	614	(16.0)		1.1	
Chicken	Millon, Lugg, Kapeller	Beach	59		4.3[b]	1.2[b]	4.6[a]
Hawk	Millon, Folin	Gurevich	272	(16.0)	2.6	1.1	
Eagle	Millon, Folin	Gurevich	272	(16.0)	2.6	1.2	
Pheasant	Millon, Folin	Gurevich	272	(16.0)	2.5	1.0	
Parrot	Millon, Folin	Gurevich	272	(16.0)	2.3	1.5	
Turtle	Millon, Lugg, Kapeller	Beach	59		4.6[b]	1.4[b]	4.3[a]
Frog	Millon, Lugg, Kapeller	Beach	59		4.7[b]	1.4[b]	4.7[a]
Mean with 2×S.E.		●		16.0	3.1±0.3	1.2±0.2	4—5[a]

[a] Per cent of phenylalanine.

[b] Omitted from Mean.

AMINO ACID COMPOSITION

MUSCLE PROTEINS

Aromatic Amino Acids in *Fish Muscle Proteins*

Calculated to 16.0 gm. N.

FISH	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Cod	Fischer, Isolation	Abderhalden	24	13.6	2.4	2.5	1.3
Cod	Millon, Lugg, Kapeller	Beach	59		4.5	1.3	4.3
Cod	Folin	Pottinger	527	(16.0)		1.1	
Menhaden	Millon, Folin, Kapeller	unpublished		11.6	3.3	1.0	4.8
Halibut	Isolation, Fischer	Osborne	493	(16.0)	2.4		3.0
Halibut	Folin	Pottinger	527	(16.0)		1.6	
Cat Fish	Folin	Pottinger	527	(16.0)		1.0	
Croaker	Folin	Pottinger	527	(16.0)		1.2	
Haddock	Folin	Pottinger	527	(16.0)		0.9	
Herring	Folin	Pottinger	527	(16.0)		1.3	
Lake Trout	Folin	Pottinger	527	(16.0)		1.2	
Mackerel	Folin	Pottinger	527	(16.0)		1.4	
Mullet	Folin	Pottinger	527	(16.0)		1.4	
Pilchard	Folin	Pottinger	527	(16.0)		1.3	
Red Snapper	Folin	Pottinger	527	(16.0)		1.2	
Salmon	Folin	Pottinger	527	(16.0)		1.3	
Salmon	Millon-Lugg, Kapeller	Beach	59		4.4	1.4	4.5
Shad	Folin	Pottinger	527	(16.0)		1.0	
Sea Trout	Folin	Pottinger	527	(16.0)		1.0	
Tuna	Folin	Pottinger	527	(16.0)		1.2	
Boneto	Rhode-May	Tomiyama	614	(16.0)		1.2	
Whale	Rhode-May	Tomiyama	614	(16.0)		1.2	
Sardine	Rhode-May	Tomiyama	614	(16.0)		1.4	
Unknown-Myosin	Millon-Lugg	Bailey	44	16.6	4.1	0.9	
Unknown	Rhode-May	Jones	342	(16.0)		1.3	
Mean with 2 ×S.E.					4[a]	1.3±0.1	4–5

[a] Probably.

MUSCLE PROTEINS

Aromatic Amino Acids in *Crustacean Proteins*

Calculated to 16.0 gm. N.

SPECIES	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
Clam	Folin	Pottinger 527	(16.0)		1.2	
Crab	Folin	Pottinger 527	(16.0)		1.1	
Lobster-myosin	Millon-Lugg	Bailey 44	16.1	3.5	0.8	
Shrimp	Folin	Pottinger 527	(16.0)		1.0	
Shrimp	Millon-Lugg, Kapeller	Beach 59		4.7	1.2	4.8
Scallop	Isolation, Fischer	Osborne 496	17.1	1.8		4.6

MUSCLE PROTEINS

Muscle proteins from a wide variety of species show little if any significant differences in their content of the aromatic amino acids. It may be assumed that human muscle proteins likewise contain approximately four per cent of tyrosine, 1 per cent of tryptophane, and 4 per cent of phenylalanine.

PLANT PROTEINS

Aromatic Amino Acids in *the Proteins of Autotropic Organisms (Algae, Fern, etc.)*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	TYROSINE	TRYPTO-PHANE	PHENYL-ALANINE
			gm.	gm.	gm.
Phormidium	Millon, Folin, Isolation	Mazur 442	3.4	0.2	2.1
Ulva	Millon, Folin, Isolation	Mazur 442	0.0	0.6	4.3
Laminaria	Millon, Folin, Isolation	Mazur 442	3.9	1.3	1.9
Sargassum	Millon, Folin, Isolation	Mazur 442	2.9	1.8	0.6
Gloeotrichia	Millon, Folin	Mazur 443	1.9	0.4	
Macrocystis	Millon, Folin	Mazur 443	0.6	0.6	
Lessoniopsis	Millon, Folin	Mazur 443	1.2	2.2	
Fucus	Millon, Folin	Mazur 443	1.7	0.6	
Cystoseira	Millon, Folin	Mazur 443	1.9	0.9	
Egregia	Millon, Folin	Mazur 443	1.4	1.1	
Caulerpa	Millon, Folin	Mazur 443	2.7	2.2	
Codium	Millon, Folin	Mazur 443	1.0	0.5	
Chondrus*	Millon, Folin, Isolation	Mazur 442	4.8	1.9	2.8
Diatoms	Millon, Folin	Mazur 443	0.4	7.3	
Pteridium	Millon-Lugg	Lugg 433A	4.3	1.3	

* Irish Moss.

PLANT PROTEINS

Aromatic Amino Acids in *Biologically Active Substances*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
Gramicidin	Millon, Folin, Kapeller	Christensen 162	14.6	0.0	38.6	0.0
Gramicidin	Rhode-Bates	Christensen 162	14.6		36.7	
Gramicidin	Rhode-May	Hotchkiss 310	14.8		43.7	
Tyrocidine	Millon, Folin, Kapeller	Christensen 162	14.5	14.7	5.5	24.0
Tyrocidine	Rhode-Bates	Christensen 162	14.5		7.0	
Tyrocidine	Rhode-May	Hotchkiss 310	14.3		18.6	
Yellow Enzyme	Millon, Voisenet, Kapeller	Kuhn 393	16.3	7.7	4.8	5.6
Allergen-cottonseed	Millon, Hopkins	Spies 585	19.8	1.4	0.0	
Allergen-cottonseed	Millon, Hopkins	Spies 585	20.2	1.4	0.0	
Allergen-cottonseed	Millon, Hopkins	Spies 585	11.6	1.8	0.0	
Crystalline Wheat		Balls 48	17.4	3.0		

PLANT PROTEINS

Aromatic Amino Acids in *Corn* (*Zea Mays*) Kernel Proteins other than Zein

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
Corn-white, whole	Millon, Folin, Kapeller	unpublished		5.6	0.6	5.0
Corn-white, whole	Millon, Rhode-May	Csonka 181		7.1	0.4	
Corn-yellow, whole	Millon, Kapeller	unpublished		5.5		4.0
Corn-yellow, whole	Millon-Lugg	unpublished			1.1	
Corn-yellow, whole	Millon, Rhode-May	Csonka 181		6.3	0.5	
Gluten-white	Millon, Folin, Kapeller	unpublished	10.9	5.9	0.5	6.8
Gluten-white	Millon-Lugg	unpublished	10.9	6.1	0.7	
Gluten-yellow	Millon, Folin, Kapeller	unpublished	12.7	6.7	0.7	6.4
Gluten-meal	Millon, Folin, Kapeller	unpublished		7.1	1.5	6.7
Gluten	Rhode-May	May 440			1.1	
Gluten-NaOH soluble	Isolation, Fischer	Osborne 490	(16.0)	3.8		1.7
Glutelin	Millon, Rhode-May	Csonka 177	(16.0)	5.0	2.1	
Germ-white	Millon, Folin, Kapeller	unpublished	11.8	4.3	1.3	5.6
Germ-white	Millon-Lugg	unpublished	11.8	3.8	1.3	
Germ-yellow	Millon, Folin, Kapeller	unpublished	12.8	6.7	1.3	5.5
Zein Residue	Millon-Lugg, Kapeller	unpublished	10.9	6.2	1.1	4.5
Albumins-yellow	Millon-Lugg, Kapeller	unpublished	12.6	3.8	0.7	1.7
Bran-yellow	Millon-Lugg	unpublished		10.8	1.0	

PLANT PROTEINS

Aromatic Amino Acids in *Edestin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE	NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE	
		per cent	gm.	gm.	gm.	
Fischer	Abderhalden 1	18.6			2.1	
Isolation, Fischer	Abderhalden 8	(18.6)			3.4	
Millon-Lugg	Bailey 44	18.4	3.7	1.3		
Millon, Folin	Beach 55	17.1	3.9	1.2		
Millon, Folin	Folin 231	(18.6)	5.0	1.2		
Millon, Folin	Folin 232	(18.6)	3.9	1.3		
Millon, Folin	Folin 233	(18.6)	3.7	1.3		
Voisenet	Fürth 249	(18.6)		1.5		
Millon-Fürth	Fürth 250	(18.6)	3.7			
Millon-Arnow	Gordon 261		2.7			
Rhode-May	Jones 342	(18.6)		2.2		
Kapeller-Adler	Kapeller 350	(18.6)			3.4	
Millon, Voisenet	Kiesel 363	18.4	1.9	1.4		10% NaCl extraction
Millon, Voisenet	Kiesel 363	18.4	1.9	1.1		3% NaCl extraction
Millon, Voisenet	Kiesel 363	18.4	3.2	1.4		heat coagulum
Millon, Voisenet	Kiesel 363	18.4	3.3	0.9		heat coagulum
Millon, Voisenet	Kiesel 363	18.4	2.7	1.3		heat filtrate
Millon	Kiesel 363	18.4	2.6			heat filtrate
Kollmann	Kollmann 376	(18.6)			3.0	
Voisenet-Kraus	Kraus 389	(18.6)		1.1		
Millon-Lugg	Lugg 432	18.5	3.7	1.3		
Rhode-May	May 440	(18.6)		1.3		
Folin	May 440	(18.6)		1.2		
Hopkins-Shaw	Shaw 577	(18.6)		1.1		
Rhode-May	Shaw 577	(18.6)		2.2		
Rhode-May	Tomiyama 614	(18.6)		1.3		
Millon	Zuwerkalow 699	(18.6)	4.2			
Mean with 2×S.E.			3.3±0.4	1.4±0.1	>4	

PLANT PROTEINS

Aromatic Amino Acids in *Gliadin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	TYROSINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
Isolation, Fischer	Abderhalden	7	(17.7)	2.2		2.4
Millon, Folin	Folin	231	(17.7)	3.2	1.0	
Millon, Folin	Folin	232	(17.7)	2.8	0.8	
Millon, Folin	Folin	233	(17.7)	3.0	0.7	
Pauly-Hanke	Hanke	280	(17.7)	2.1		
Millon, Folin	Holiday	302	(17.7)	2.8	0.9	
Spectrophotometric	Holiday	302	(17.7)	3.5	0.6	
Rhode-May	Jones	342	(17.7)		0.8	
Voisenet-Kraus	Kraus	389	(17.7)		0.5	
Rhode-May	May	440	(17.7)		1.0	
Folin	May	440	(17.7)		1.0	
Isolation, Fischer	Osborne	489	17.7	1.1[a]		2.2
Mean with 2 ×S.E.				2.8±0.4	0.8±0.1	

[a] Omitted from mean.

PLANT PROTEINS

Aromatic Amino Acids in *Grass Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITROGEN	TYROSINE	TRYPTOPHANE
			per cent	gm.	gm.
Cocksfoot	Millon-Lugg	Lugg 432	12.9	4.8	2.1
Cocksfoot	Millon-Lugg	Lugg 433		4.8	2.1
Canary	Millon-Lugg	Lugg 432	15.4	5.0	2.0
Rye	Millon-Lugg	Lugg 432	13.6	4.8	2.0
Rye	Millon-Lugg	Lugg 433		4.9	2.2
Meadow	Millon-Lugg	Lugg 432	14.0	4.8	2.1
Fescue	Millon-Lugg	Lugg 432	14.3	4.9	2.2
Dogtail	Millon-Lugg	Lugg 432	14.1	4.9	2.3
Lucerne	Millon-Lugg	Lugg 432	14.0	5.6	2.3
Salt bush	Millon-Lugg	Lugg 432	12.1	5.3	2.0
Mean with 2 ×S.E.				5.0±0.2	2.1±0.1

PLANT PROTEINS

Aromatic Amino Acids in *Leaf Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Alfalfa Meal	Millon, Folin, Kapeller	unpublished		10.6	5.7	3.0	4.5
Alfalfa Meal	Millon-Lugg	unpublished		10.6	.	1.6	
Clover-red	Millon-Lugg	Lugg	432	12.8	5.2	2.0	
Clover-white	Millon-Lugg	Lugg	432	13.1	5.2	2.2	
Runner Bean	Millon-Lugg	Lugg	432	13.3	4.9	1.8	
Spinach	Millon-Lugg	Lugg	432	14.1	5.4	1.9	
Beet Tops	Millon-Lugg	Lugg	433		5.3	2.4	
Mean with 2 ×S.E.					5.3±0.2	2.1±0.4	4 to 5

PLANT PROTEINS
Aromatic Amino Acids in *Miscellaneous* Plant Proteins

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Cottonseed-globulin	Millon, Folin	Folin	233	(18.6)	3.1	1.1	
Cottonseed-globulin	Millon-Lugg, Kapeller	Fontaine	238	16.9	3.4*	1.3*	7.8
Cottonseed-globulin	Millon-Lugg, Kapeller	Fontaine	238	17.9	2.9	1.3	8.1*
Cottonseed-globulin	Rhode-May	Jones	342	(18.6)		2.2	
Cottonseed-globulin	Isolation, Fischer	Abderhalden	6	(18.6)	2.0		3.4
Cottonseed-meal	Millon-Lugg, Kapeller	unpublished			3.2	1.3	6.8
Linseed meal	Millon, Folin, Kapeller	unpublished			5.1	3.0	5.6
Linseed meal	Millon-Lugg	unpublished			5.1	1.9*	
Peanut-Arachin	Millon, Kapeller	unpublished		17.0	4.1		5.5
Peanut-Arachin	Folin-Denis	Johns	322	18.3	4.8		
Peanut-Arachin	Rhode-May	Jones	342	(18.3)		0.8	
Peanut meal	Millon-Lugg, Kapeller	unpublished			4.4	1.0	5.4
Soybean Meal (?)	Millon-Lugg, Kapeller	Heinrich	286	?	5.6		3.6
Soybean meal	Millon, Folin, Kapeller	unpublished			4.1*	1.6	5.7
Soybean glycinin	Rhode-May	Jones	342	(17.5)		1.6	
Soybean glycinin	Millon, Voisenet	Kiesel	362	17.5	1.8	1.6	
Soybean glycinin	Rhode-May	May	440	(17.5)		1.5*	
Soybean protein	Rhode-May	Tomiyama	614	(16.0)		1.3	
Castorbean-Ricin	Isolation, Voisenet	Karrer	355	(17.0)	2.7	0.4	
Barley-Hordein	Isolation	Kleinschmitt	369	17.2	3.7		5.1
Barley-Hordein	Voisenet-Kraus	Kraus	389	(17.2)		0.6	
Pea (?)-Legumin	Kollman	Kollman	376	(17.0)			4.6
Squashseed-glob.	Voisenet-Kraus	Kraus	389	(17.0)		1.8	
Cucubitseed-glob.	Millon, Folin	Vickery	657	18.5	3.6	1.5	
Rubber latex	Millon-Lugg	Tristram	620	15.0	6.7	1.4	
Flaxseed meal	Millon-Lugg, Kapeller	unpublished			3.9	1.6	6.0
Lupine Meal ?		Heinrich	286	?	5.6		4.5
Oat Meal	Millon-Lugg, Kapeller	unpublished			4.5	1.3	7.2
Rice Cereal	Millon-Lugg, Kapeller	unpublished				1.3	6.3

* Best Values

PLANT PROTEINS
Aromatic Amino Acids in *Oat* and *Rice* Proteins

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		TYROSINE	TRYPTOPHANE
OAT PROTEINS				gm.	gm.
Whole-Rockland	Millon, Rhode-May	Csonka	182	12.0	0.3
Whole-Commercial	Millon, Rhode-May	Csonka	182	7.4	0.4
Rolled	Millon, Rhode-May	Csonka	182	2.9	1.5
Middlings	Millon, Rhode-May	Csonka	182	4.7	
Shorts	Millon, Rhode-May	Csonka	182	6.7	
Glutelin	Millon, Rhode-May	Csonka	177	4.4	1.9
RICE PROTEINS					
Whole	Rhode-May	Kik	364		1.0
Polished	Rhode-May	Kik	364		1.0
Germ	Rhode-May	Kik	364		0.7
Polishings	Rhode-May	Kik	364		0.9
Arkansas 155	Rhode-May	Kik	364		0.7
Shoemed	Rhode-May	Kik	364		1.1
Arcadia	Rhode-May	Kik	364		1.1
Zenith	Rhode-May	Kik	364		0.9
Fortuna	Rhode-May	Kik	364		1.1
Glutelin	Millon, Rhode-May	Csonka	177	5.8	1.8

PLANT PROTEINS

Aromatic Amino Acids in *Viruses*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ANALINE
				per cent	gm.	gm.	gm.
Virus-Tobacco Mosaic	Millon, Hopkins, Kapeller	Knight	370	(16.0)	3.8	4.5	6.0
Virus-Tobacco Mosaic	Millon-Lugg, Kapeller	Ross	556	(16.0)	3.9	2.0	6.3
Virus-Tobacco Mosaic	Hopkins-Shaw	Ross	556	(16.0)		4.5	
Virus-Tobacco Mosaic	Millon, Hopkins	Ross	557	15.9	4.3	4.9	6.7
Virus-Yellow Ancuba	Millon, Hopkins, Kapeller	Knight	370	(16.0)	3.9	4.2	6.3
Virus-Green Ancuba	Millon, Hopkins, Kapeller	Knight	370	(16.0)	3.9	4.2	6.1
Virus-Holme's ribgrass	Millon, Hopkins, Kapeller	Knight	370	(16.0)	6.4	3.5	4.3
Virus-Holme's mashed	Millon, Hopkins, Kapeller	Knight	370	(16.0)	3.9	4.3	6.1
Virus-J14D1	Millon, Hopkins, Kapeller	Knight	370	(16.0)	3.8	4.4	6.1
Virus-Cucumber 4	Millon, Hopkins, Kapeller	Knight	370	(16.0)	3.8	1.4	10.2
Virus-Cucumber 3	Millon, Hopkins, Kapeller	Knight	370	(16.0)	4.0	1.5	10.0

PLANT PROTEINS

Aromatic Amino Acids in *Wheat Proteins* other than Gliadin

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYPTO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Whole Wheat	Millon, Kapeller	unpublished			4.8		5.7
Whole Wheat	Millon-Lugg	unpublished			3.9	1.2	
Hard wheat	Millon, Rhode-May	Csonka	179		4.2	0.4	
Hard wheat	Millon, Rhode-May	Csonka	179		3.5	0.4	
Soft wheat	Millon, Rhode-May	Csonka	179		2.8	0.5	
Wheat Flour	Millon, Folin, Kapeller	unpublished		12.8	3.8	0.8	5.5
Hard wheat flour	Millon, Rhode-May	Csonka	180		2.5	1.3	
Hard wheat flour	Millon, Rhode-May	Csonka	180		3.7	1.4	
Wheat Gluten	Padoa	508		?	1.3	1.2	4.1
Glutelin	Millon, Rhode-May	Csonka	177	(16.0)	5.4	2.1	
Glutenin	Millon, Folin	Folin	231	(16.0)	4.6	1.7	
Glutenin	Rhode-May	Jones	342	(17.5)		1.6	
Glutenin	Rhode-May	May	440	(17.5)		1.6	
Glutenin	Folin	May	440	(17.5)		1.5	
Glutenin	Isolation, Fischer	Osborne	489	17.5	3.9		1.8
Germ	Millon, Folin, Kapeller	unpublished			3.8	1.0	4.2
Germ-Leucosin	Isolation, Fischer	Osborne	489	16.8	3.1		3.6
Bran	Rhode-May	Jones	342	(16.0)		4.8	

PLANT PROTEINS
Aromatic Amino Acids in *Yeast* and *Mold Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	TYRO-SINE	TRYP-TO-PHANE	PHENYL-ALANINE
				per cent	gm.	gm.	gm.
Brewer's	Millon, Folin, Kapeller	unpublished			4.0	1.4	3.5
Brewer's	Millon, Folin, Kapeller	unpublished		14.4	3.6	0.9	4.3
Brewer's	Millon, Folin, Kapeller	unpublished			4.5	0.8	4.5
Brewer's	Millon, Folin, Kapeller	unpublished			4.8	1.1	4.1
Brewer's-H₂O soluble	Millon, Rhode-May	Csonka	178	15.3	4.3	2.8	
Brewer's-NaCl soluble	Millon, Rhode-May	Csonka	178	16.2	3.8	2.5	
Brewer's-NaOH soluble	Millon, Rhode-May	Csonka	178	16.4	3.8	1.6	
Baker's	Millon, Folin, Kapeller	unpublished			4.2	1.1	3.9
Baker's-H₂O soluble	Millon, Rhode-May	Csonka	178	15.6	4.9	2.7	
Baker's NaCl soluble	Millon, Rhode-May	Csonka	178	16.2	4.2	3.0	
Baker's NaOH soluble	Millon, Rhode-May	Csonka	178	14.8	3.4	1.9	
Steep Water	Millon, Kapeller	unpublished			4.9		4.1
Mold-Aspergillus	Isolation	Woolley	689	5.15	1.3*	0.1*	0*
Mean with 2×S.E.					4.2±0.2	1.8±0.5	4.1
* Omitted from mean							

PLANT PROTEINS
Aromatic Amino Acids in *Zein*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	TYROSINE	TRYPTO-PHANE	PHENYL-ALANINE
			per cent	gm.	gm.	gm.
Isolation	Brazier	129	17.5	2.4*		6.9
Isolation	Dakin	186	16.1			7.6
Folin	Folin	232	(16.1)		0.2	
Millon, Folin	Folin	233	(16.1)	5.9	0.2	
Pauly-Hanke	Hanke	280	(16.1)	3.6		
Spectrophotometric	Holiday	302	(16.1)	8		
Kapeller-Adler	Kapeller	350	(16.1)			5.0
Kollmann	Kollmann	376	(16.1)			6.5
Voisenet-Kraus	Kraus	388	(16.1)		0.2	
Pucher-Arhimo	Laine	398	(16.1)	5.4		
Rhode-May	May	440	(16.1)		0.0	
Folin	May	440	(16.1)		0.0	
Isolation, Fischer	Osborne	490	16.1	3.6		4.9
Isolation, Fischer	Osborne	499	16.1	3.6		6.6
Kapeller-Adler	Virtanen	663	14.5			7.5
Millon-Lugg, Kapeller	unpublished		15.4	5.2	0.1	6.4
Mean with 2×S.E.				5.0±1.2	0.1	6.4±0.7
* Omitted from mean						

PLANT PROTEINS

Autotropic-Organisms: The tyrosine and tryptophane analyses were carried out by Mazur and Clark (442, 443) on the whole lipid-free organisms. In a typical experiment, 50 gm. of *Sargassum* were suspended in 500 ml. of 10 per cent barium hydroxide and heated on the steam bath for 4 days. The barium was removed and the solution was concentrated to 100 ml. After treatment with kaolin, a 20 ml. aliquot was found to contain 1.32 mg. of tyrosine nitrogen or approximately 0.17 per cent tyrosine in the dried *Sargassum* and 1.39 mg. of tryptophane nitrogen or 0.1 per cent of tryptophane on the dried basis.

In spite of the fact that Homer (305) and Onslow (483) showed that tryptophane was destroyed on long heating with baryta, especially in the presence of certain inorganic salts; that Fujiwara and Kataoka (254) and Schild and Enders (566) proved that the Folin phosphomolybdotungstic acid method for tryptophane gave entirely unreliable results except with purified proteins and amino acid mixtures; that Wu (693) found that one part of cuprous copper in 5,000,000 can reduce the Folin phenol reagent, and that Herriott (289) found that small quantities of cupric ion can greatly enhance the quantity of blue color formed by many substances which may be found in the hydrolysate of a highly impure protein preparation, nevertheless, Mazur and Clarke (442) say "The analytical method employed in this study gave quantitatively reliable values for tyrosine and tryptophane . . . . " The figures given in the table, especially those for tryptophane, are very likely the resultant of a number of extraneous substances some of which tend to destroy tryptophane and others which reduced Folin's reagent to give "high" values. If their results were true, the protein $(N \times 6.25)$ of diatoms should be a most valuable commercial source of tryptophane, especially for animal feeds. Lugg's (433A) values appear reasonable.

Biologically Active Substances: The large quantity of tryptophane present in the bacteriocidal polypeptide, gramicidin and the lack of tyrosine in this substance are noteworthy. In the case of the tyrocidine, the quantity of tyrosine is considerable and equal to or greater than that of tryptophane.

It should be noted that two investigators using minor modifications of the Voisenet-Rhode reaction obtained large differences in the tryptophane "content" of apparently identical, highly purified protein-like substances.

Corn Proteins (except Zein): Most corn proteins appear to be a good source of tyrosine and phenylalanine but except for the germ

proteins are somewhat deficient in tryptophane. Corn gluten and corn albumins (steep water proteins) are the poorest in tryptophane, while the germ proteins and the zein-free gluten (zein residue) appear to be average sources of this essential amino acid. The large quantity of tyrosine in corn bran proteins requires confirmation.

Edestin: The tyrosine values are of special interest in Kiesel's experiments on the effects of heat coagulation of proteins. Heat coagulation is rather generally used to prepare a protein for analysis. These results and those of Calvery on pepsin indicate that proteins so prepared may differ somewhat in composition when compared to the original crystalline material.

Grasses and Green Leaf Proteins: These tissue proteins, in contrast to many endosperm proteins, appear to be good sources of tryptophane.

Seed Proteins: Cottonseed globulin, linseed meal, soybean meal, squash seed globulin, cucubit seed globulin, and flaxseed meal proteins contain more tryptophane than corn and wheat proteins. The principal protein of the peanut, arachin, appears to be quite deficient in tryptophane.

Oat and Rice Proteins: These proteins appear to yield more tryptophane than wheat or corn. Csonka's tyrosine values in oat proteins require confirmation.

Viruses: A number of the plant viruses are unusually rich in tryptophane, as determined by the Shaw-McFarlane modification of the Hopkins-Cole procedure. The large quantity of phenylalanine in cucumber virus is noteworthy.

Wheat Proteins (other than Gliadin): Wheat proteins do not contain a superabundance of the aromatic amino acids and many of them are deficient in tryptophane.

The high value for tryptophane in wheat bran warrants further study.

Yeast Proteins: Although the quantity of tyrosine and of phenylalanine present in yeast proteins appears to be approximately constant, further study is necessary to ascertain the quantity of tryptophane in the total protein. It seems to be between 1 and 2 per cent.

Zein: This protein is devoid or almost devoid of tryptophane, a fact long known. Zein is relatively rich in both tyrosine and phenylalanine.

TISSUE PROTEINS

Aromatic Amino Acids in *Miscellaneous Beef Tissue and Organ Proteins*
(unpublished experiments)

Calculated to 16.0 gm. N.

ORGAN	METHOD	NITROGEN	TYROSINE	TRYPTO-PHANE	PHENYL-ALANINE
		per cent	gm.	gm.	gm.
Kidney	Millon-Lugg, Kapeller	15.6	3.2	1.2	5.5
Kidney[a]	Millon, Folin		4.8	1.7	
Kidney[b]	Millon-Lugg, Kapeller		4.6	1.8	5.5
Lung	Millon-Lugg, Kapeller	15.3	2.7	0.8	4.7
Lung[b]	Millon-Lugg, Kapeller		3.8	1.8	4.1
Pancreas	Millon-Lugg, Kapeller	15.5	3.0	1.4	4.4
Salivary Gland	Millon-Lugg, Kapeller	15.7	2.6	0.8	3.6
Spleen	Millon-Lugg, Kapeller	15.7	2.9	0.8	4.6
Thymus	Millon-Lugg, Kapeller	15.4	2.5	0.6	3.3
Ovaries	Millon-Lugg, Kapeller	15.8	2.6	0.7	4.8
Testes	Millon-Lugg, Kapeller	15.4	3.1	1.0	5.5
Heart	Millon-Lugg, Kapeller	14.8	3.4	1.1	5.7
Heart[b]	Millon-Lugg, Kapeller		4.4	1.4	5.1
Bladder	Millon-Lugg, Kapeller	15.9	2.9	0.6	3.5
Intestine	Millon-Lugg, Kapeller	15.3	3.3	0.7	4.8
Adrenal	Millon-Lugg	15.9	3.7	1.1	
Stomach[b]	Millon-Lugg, Kapeller		3.7	1.0	3.3
[a] Lee 411					
[b] Beach 59					

TISSUE PROTEINS

The relatively small quantity of tryptophane present in many of the glandular tissues does not appear to be the result of any experimental error as similar values were found by the Folin procedure. The deficiency in tryptophane is apparently the result of the quantity of albuminoids and of nucleo-proteins present in these organs for the recent results of Mirsky (461) suggest that "pure" nucleoproteins are devoid of this amino acid.

THE SULFUR CONTAINING AMINO ACIDS*

CYSTINE, CYSTEINE, AND METHIONINE

	Cystine	Cysteine	Methionine
Empirical Formula	$C_6H_{12}O_4N_2S_2$	$C_3H_7O_2NS$	$C_5H_{11}O_2NS$
Optical Form	l	l	l
Molecular Weight	240.23	121.12	149.15
Carbon	29.97		40.23
Hydrogen	5.03		7.43
Nitrogen	11.66		9.39
Oxygen	26.64		21.45
Sulfur	26.69		21.50
Melting Point	256–258 (decomp.)		283 (uncor.)

PART I

HYDROLYSIS

CYSTINE AND CYSTEINE: The destructive effects of alkali on cystine and cysteine are so extensive as to preclude the use of these reagents as hydrolyzing agents of proteins when either of these two amino acids *per se*, are to be determined.

The effects of acid hydrolyzing agents on cystine and cysteine are variable. Bolling and Block (112) found no loss of cystine as indicated by recovery experiments during 20 hour hydrolysis of β-lactoglobulin with 10 volumes of a 1:1 mixture of 18 per cent hydrochloric acid and 90 per cent formic acid. The non-specific Folin phospho-18-tungstic acid and the specific Fleming-Vassel p-phenylene-diamine methods were used to determine cystine.

Lugg (428), using a modification of the Folin method, likewise found very slight losses of cystine and only a small loss of cysteine during HCl hydrolysis in the absence of carbohydrate. In the presence of small amounts of carbohydrate, 6 to 7 per cent of cystine was lost while 85 to 100 per cent of the cysteine could not be recovered. When relatively large quantities of carbohydrate were present, even cystine was largely destroyed.

The destructive action of carbohydrate breakdown products on cysteine and cystine may be explained, in part at least, by the formation of derivatives of thiazolidine carboxylic acid. Ratner

* Recommended procedures are starred.

142

and Clarke (540) have shown that formaldehyde reacts with cysteine to yield thiazolidine-4-carboxylic acid.

$$
\begin{array}{ccc}
CH_2SH & & CH_2 \text{---} S \\
| & \bullet & | \quad\quad\quad\diagdown \\
& & \quad\quad\quad CH_2 \\
& & \quad\quad\quad\diagup \\
CHNH_2+HCHO & \rightleftarrows & CH\text{---}NH \quad\quad +H_2O \\
| & & | \\
COOH & & COOH
\end{array}
$$
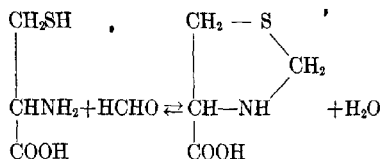
Pollard and Chibnall (524), using the highly specific Sullivan reaction, found comparable yields of cystine in grass proteins following hydrolysis with 20 per cent HCl overnight and peptic, tryptic, and ereptic digestion. However, 20 hour hydrolysis with 8 N H_2SO_4 gave only 84 per cent of the expected quantity of cystine.

Bailey (43), using modifications of the Folin and Sullivan cystine methods, found that cystine added to edestin gave 99 ± 2 per cent recoveries, but the *absolute* cystine values varied, indicating that part of the cystine in peptide linkage may be more readily destroyed. He reports that 30 per cent of the cystine and 20 per cent of the methionine present could be lost on hydrolysis with 5 N HCl for 15 hours.

Miller and duVigneaud (455) found that when insulin, a protein devoid of carbohydrate, was hydrolyzed with an equal mixture of concentrated HCl and formic acid for 48 hours there was little or no destruction of cystine. If the hydrolysis was carried out for the same length of time with 20 per cent HCl alone, an appreciable loss was observed. It should be pointed out however that 6 to 8 hour hydrolyses with 18 to 20 per cent HCl are entirely adequate for all protein preparations which we have investigated.

A number of investigators have shown that abnormally high results for cystine were obtained when the original Folin phospho-18-tungstic acid method was applied to deaminated or partially hydrolyzed proteins or to preparations hydrolyzed in the presence of large quantities of carbohydrate. Results so obtained must therefore be accepted with great caution.

Alving and Mirsky (30) made the interesting observation that if a protein (serum albumin, serum globulin) is hydrolyzed with different concentrations of sulfuric acid (6 to 11 N), the "cystine" content of the protein, as estimated by the Folin method, increases with increasing concentration of the acid.

McFarlane, Fulmer, and Jukes (448) found that the amount of cystine in casein, egg albumin, and egg yolk proteins was approximately 20 per cent higher following tryptic digestion than by sul-

furic acid hydrolysis. Folin's phospho-18-tungstic acid method was used for the estimations.

Lugg (431) refluxed the protein with an excess of 57 per cent HI under nitrogen for 6 to 8 hours. The hydrolytic losses are minimized in this strongly reducing atmosphere. Except for technical disadvantages, hydriodic acid appears to be the most suitable *hydrolyzing agent for use with protein preparations which contain carbohydrate and similar compounds. It is usually unnecessary in the determination of cystine in protein preparations of adequate nitrogen content.*

Probably the most careful series of investigations on the hydrolytic destruction of cystine are those of Sullivan, Hess and their collaborators. Sullivan, Hess, and Smith (600) found somewhat less cystine in insulin hydrolysates after hydrolysis with 20 per cent hydrochloric acid than after heating with 20 per cent HCl in 90 per cent formic acid for 12 hours (oil bath, 125 to 135°). They stress the point that if hydrolysates are kept for some time there is a significant loss of cystine as estimated by Brdicka's polarographic method or by Sullivan's colorimetric procedure.

Hess, Sullivan, and Palmes (294) investigated the hydrolytic losses of cystine in a carbohydrate containing protein (tobacco mosaic virus). The Sullivan colorimetric and the Mörner-Okuda iodine titration techniques for estimating cystine were used. Their results are summarized below:

Hydrolyzing Agent	Time of Heating hours	Per Cent of Cystine	
		Colorimetric	Titrimetric
20% HCl	6	0.47	0.48
20% HCl in Formic Acid	24	0.53	0.48
6 N H$_2$SO$_4$ under N$_2$	12	0.67	0.71
20% HCl plus 1% TiCl$_2$	2	0.64	0.64
57% HI under N$_2$	18	0.73	0.70
concentrated HI-HCOOH under N$_2$	24	0.69	0.69

The value of hydrolyzing the protein in the presence of certain strongly reducing substances to prevent humin formation is self evident.

Methionine: Bailey (43) reported that 20 per cent of the methionine present in a protein can be lost during hydrolysis in the presence of large quantities of carbohydrate.

Losses during demethylation with HI will be mentioned later in this chapter.

CHAPTER III

PART II

THE DETERMINATION OF CYSTINE AND CYSTEINE

1. THE DETERMINATION OF CYSTINE FROM THE LABILE SULFUR IN PROTEINS (SCHULZ)

Historical: G. J. Mulder (467) in 1838 was the first to show that if proteins were heated with alkali and a heavy metal salt, the metal sulfide was formed. Fleitmann, 10 years later (226) pointed out that only a portion of the sulfur of proteins is precipitated as the metallic sulfide under these conditions. This finding suggested the presence of at least two forms of sulfur in the protein molecule.

Numerous other investigators studied the alkali labile sulfur in proteins among whom was Schulz (573) who found that if cystine or cysteine were heated for 10 to 25 hours with an excess of zinc filings and 30 per cent sodium hydroxide in the presence of lead acetate or bismuth oxide, one half of the total sulfur could be found in the precipitate after the solution had been acidified with acetic acid according to Fleitmann.

These results were generally interpreted as showing that only one half of the sulfur of cysteine was alkali labile. However, although only 52 to 54 per cent of the total S of cystine and cysteine were found in the precipitate under the conditions mentioned above, Schulz says (573) "Die andere Hälfte des Schwefels ist nach Abspaltung der ersten Hälfte in einer solchen Form vorhanden, dass sie durch Säure als Schwefelwasserstoff abgespalten werden kann."

A. Clarke's Modification of the Schulz Procedure (164, 695, 111)

Principle: Cystine is reduced to cysteine by zinc in acid solution. An excess of lead acetate and sodium hydroxide is added, and on prolonged heating, cysteine yields all its S as PbS.

Method: To 5 ml. of a protein hydrolysate containing 0.5 to 1.0 mg. of cystine S, add 4 to 6 times the calculated quantity of zinc filings and 1 ml. of N HCl. Heat on a water bath to dissolve the Zn. Then add 0.2 ml. of 10 per cent lead acetate and 2 ml. of 20 per cent NaOH. Seal the tube and heat at 90 to 95° for 24 hours or longer. Centrifuge, remove the supernatant liquid and repeat the heating with alkaline plumbite if necessary. Wash the PbS with 1 per cent NaOH.

145

Oxidize the PbS to SO_4^{++} with 500 mg. of $KClO_3$ followed by 4 to 5 ml. of a cold solution of 10 ml. of bromine in 150 ml. of concentrated HCl diluted with 100 ml. of water. Stir until the black color of PbS has disappeared. Then add 1 to 2 ml. of concentrated HCl and warm on the steam bath to remove all Cl_2. Evaporate to dryness. Extract the residue twice with 20 ml. portions of hot 20 per cent HCl to separate from any SiO_2. Evaporate the extract to dryness. Dissolve the residue in 50 ml. of 0.1 N HCl and precipitate the sulfate with $BaCl_2$ as usual.

Comment: In spite of Schulz's (573) finding that only one half the sulfur of cystine and cysteine was precipitated under his conditions, a comparison of his results on "abspaltbarem Schwefel" formed from horse serum albumin, serum globulin, crystalline horse oxyhemoglobin, and other proteins is most revealing.

Protein	Cystine from Labile S (Schulz)	Cystine from Recent Data in the Literature
	per cent	per cent
Serum albumin	4.8	5.0 ± 0.3
Serum globulin	2.4	2.5 ± 0.3
Oxyhemoglobin (Horse)	0.7	0.7
Globin	0.8	0.8
Egg Albumin	1.8	1.8

It thus appears that Schulz's zinc-alkaline plumbite method published in 1898 should be classified as the first quantitative procedure for the determination of any amino acid in proteins. The method was in fact published one year before the isolation of cystine from proteins by Mörner and by Embden.

It appears to the authors that the Schulz decomposition of cystine to sulfide could be used for the colorimetric determination of cystine by coupling the H_2S liberated on acidification with dimethyl-p-phenylene-diamine to yield methylene blue. This method should be of particular value when the cystine determinations have to be carried out in the presence of large quantities of carbohydrate.

2. REDUCTION OF PHOSPHOTUNGSTIC ACID
(WINTERSTEIN, 686; FOLIN, 231)

Historical: In 1901, E. Winterstein (686) reported that when a solution of cysteine is added to dilute phosphotungstic acid, a white precipitate is formed which consists solely of cystine. The phosphotungstic acid, which is reduced, turns a very dark blue.

The reaction between cysteine and phosphotungstic acid was employed by Folin and Looney (231) in 1922 as the basis for the quantitative estimation of cystine in solution. They used Heffter's (285) observation that cystine was reduced to cysteine by Na_2SO_3. This reduction, which Folin believed to be quantitative, was shown by Clarke (165) to take the following course.

$$RS \cdot SR + Na_2SO_3 \rightarrow RS \cdot Na + RS \cdot SO_3Na$$
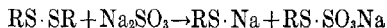
The sodium salt of cysteine reduced the phospho-18-tungstic acid (Uric Acid Reagent) to yield lower oxides of tungsten. Wu (693) pictures the reduction as follows:

$$18 \overset{H_2}{WO_3} \rightarrow 16 \ WO_3 \cdot W_2O_5 \cdot H_2O$$
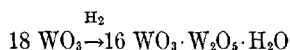
A. The Method of Folin and Looney (231)

Reagents: Phospho-18-tungstic Acid, according to Folin and Macallum (228). 100 gm. of Na_2WO_4 are boiled with 80 ml. of 85 per cent H_3PO_4 and 750 ml. of water for several hours. After cooling the solution is diluted to 1 liter.

Method: 1 to 5 gm. of protein are hydrolyzed under reflux with 25 ml. of 20 per cent H_2SO_4 for 12 hours. The hydrolysate is diluted to 100 ml. Aliquots (1 to 10 ml.) are pipetted into 100 ml. volumetric flasks. 20 ml. of saturated Na_2CO_3 and 10 ml. of freshly prepared 20 per cent Na_2SO_3 are added. The solution is mixed. After standing for 5 minutes, 3 ml. of phospho-18-tungstic acid (uric acid reagent) are added with swirling. After 10 minutes, the solution is diluted to the mark and read against a cystine standard prepared in the same way.

Comment: This procedure of Folin and Looney has been much criticized and often modified, but it is still the real basis for all the cystine and cysteine methods which depend on the reduction of hexavalent tungsten.

The chief criticisms pertain to its non-specificity towards cystine. Much of the earlier difficulties were due to the commercial sodium tungstate containing some molybdenum. The resulting phospho-molybdotungstic acid was then reduced by tyrosine and other phenolic groups in the hydrolysate (*cf.* Chapter II). The phospho-18-tungstic acid reagent is also reduced by metals (Wu, 693), carbohydrate decomposition products and many other substances which may be present in a protein hydrolysate.

The modifications given below are usually attempts to increase the specificity of the method for cystine.

B. *Folin and Marenzi's Cystine Method* (234)

Reagents: Phospho-18-tungstic Acid Reagent free of Molybdenum (235). 100 gm. of Na_2WO_4 are dissolved in 200 ml. of water. 20 ml. of 85 per cent H_3PO_4 are slowly added to the Na_2WO_4 solution with cooling. A stream of H_2S is passed through the solution at a very moderate rate for 20 minutes. Then 10 ml. more of 85 per cent H_3PO_4 are added, the flow of H_2S being continued. The solution should now be slightly acid to Congo paper. If it is not, a little more H_3PO_4 should be added. After 20 minutes, the solution is filtered. The clear liquid is then extracted in a separatory funnel with 1.5 volumes (300 ml.) of ethanol. The lower layer, filtered if necessary, is drained into a tared flask. Water is added until the contents of the flask weigh 300 gm. The H_2S is removed by boiling. The flame is reduced and 20 ml. of 85 per cent H_3PO_4 are added. The solution is refluxed for 1 hour. The condenser is removed and a few drops of bromine are added to destroy any blue color. The bromine is removed by boiling.

In a liter beaker, a solution of lithium phosphate is prepared by adding 50 ml. of 85 per cent H_3PO_4 to 25 gm. of Li_2CO_3. It is dissolved in 250 ml. of water and boiled to remove the CO_2.

The cooled Li_3PO_4 solution is added to the phosphotungstic acid and the reagent is diluted to 1 liter.

Phospho-18-tungstic Acid Reagent (Simplified Procedure (236): 100 gm. of Na_2WO_4 are added to 32 to 33 ml. of 85 per cent H_3PO_4 in 150 ml. of water. The solution is boiled very gently under reflux for 1 hour and diluted to 500 ml. Then 3 to 5 gm. of Na_2WO_4 are added and the solution is again boiled for 10 to 15 minutes. A little bromine water is added and the excess Br_2 is boiled off. The phospho-18-tungstic acid (uric acid) reagent should give a negative test with tyrosine and with urea plus sodium cyanide.

Folin (236) believes this reagent consists principally of phospho-18-tungstic acid B (Wu, 693) and is not as sensitive to phenols as that containing phospho-18-tungstic acid A.

Method: 1. Hydrolysis. 100 mg. of protein are hydrolyzed over night with 20 ml. of 6 N H_2SO_4. The solution is diluted to 100 ml. and decolorized by shaking with kaolin.

2. Determination. Two ml. of freshly prepared 20 per cent Na_2SO_3 are added to 2 ml. of the unknown. After standing for 1 minute, 18 ml. of 20 per cent Na_2CO_3 (plus 0.5 ml. for each ml. of hydrolysate over 2 ml.), 2 ml. of 20 per cent Li_2SO_4, and 8 ml. of phospho-18-tungstic acid reagent are added with mixing. After 3 to 5 minutes, the solution is diluted to 100 ml. with 3 per cent Na_2SO_3 and read.

Comment: Folin and Marenzi (234) commenting on the above procedure say "each change represents only a minor improvement, such as one would expect to come in the development of any new method." The improvements were, the preparation of a "Uric Acid Reagent" free from "Phenol Reagent" (235), the reduction of the sulfite blank by reducing the quantity of 20 per cent Na_2SO_3 from 10 ml. to 2 ml., and the use of more phosphotungstic acid reagent.

Rimington (546) added 5 ml. of 40 per cent urea before the introduction of the color reagent to prevent turbidity caused by the precipitation of the sodium salt of phospho-18-tungstic acid.

Mirsky and Anson (459) made the important observation that the color intensities given with the Folin-Marenzi uric acid reagent in the presence of sulfite by equivalent quantities of cysteine and cystine are precisely in the ratio of 2:1. The reason for this difference was explained by Clarke (165) the following year (*cf.* Historical).

C. Tompsetts' Modification of the Folin Method (615)

Principle: Na_2CO_3 used by Folin is replaced by 20 ml. of saturated $NaHCO_3$. This simple change prevents turbidity caused by sodium phosphotungstate.

D. Lugg's Adaptation of the Folin Method to Acid Solutions (425, 426)

Principle: Lugg (425) found that the color developed by the reaction of cysteine, cystine plus sulfite, etc. with phospho-18-tungstic acid is stable between *p*H 5.0 and 6.5.

If excess of the reagent is present, the color is strictly proportional to the reducer. He also found that mercuric chloride at *p*H 5.7 inhibits the formation of color by sulfhydryl and disulfide compounds but does not influence the color produced by levulinic acid or furfuraldehyde. The amount of color produced when sodium sulfite is added to the reagent alone is greater than if disulfides are present.

Reagents: 4 M Sodium Acetate: 544 gm. of $C_2H_3O_2Na \cdot 3H_2O$ are dissolved in water and diluted to 1 liter.

Citrate Buffer: 105 gm. of $C_6H_8O_7 \cdot H_2O$ and 52.5 gm. of NaOH are dissolved in 500 ml. of water with cooling. Then 13.6 gm. of $ZnCl_2$ in 20 ml. of water and 26.8 gm. of NH_4Cl in 200 ml. of water are added. The solution is diluted to 1 liter.

Buffered Sulfite: 9.5 gm. of sodium metabisulfite ($Na_2S_2O_5$) are dissolved in 70 ml. of water, 15 ml. of 4 M sodium acetate are added. The mixture is diluted to 100 ml.

0.1 M Mercuric Chloride: 2.72 gm. are diluted to 100 ml.

Mohr's Salt: 3.268 gm. of $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ are dissolved in 8.5 ml. of N H_2SO_4. Water is added to 100 ml. The solution is diluted 1 to 10 before use.

Standard Cystine: 500 mg. of cystine are dissolved in 50 ml. of N HCl. Water is added to 500 ml.

Procedure: Use 100 ml. volumetric flasks. Adjust the partially neutralized hydrolysate to *p*H 5.7 (brom cresol purple) by titration with sodium acetate.

A. Estimation of Cysteine in the Absence of Extraneous Reducers. 10 ml. of citrate buffer and 2 ml. of 4 M acetate are added in order. Then 16-x ml. of water and x ml. of hydrolysate and enough sodium acetate to bring the hydrolysate to *p*H 5.7 are added. Two ml. of Folin's phospho-18-tungstic acid reagent are then introduced. After 7 minutes, the solution is diluted to volume and read. If a step-photometer or a photoelectric colorimeter is available, filters 720 mu, 668 mu (568, 45) or 520 mu (104) should be used.

B. Estimation of Cysteine in the Presence of Extraneous Reducers. The above procedure is repeated except that 1 ml. of $HgCl_2$ solution is added prior to the introduction of the unknown.

The "true" cysteine is then given by the difference in the values obtained in A and B.

C. Estimation of Cystine in the Absence of Extraneous Reducers. The procedure given in A is used except that 1 ml. of buffered sulfite is added after the unknown.

D. Estimation of Cystine in the presence of Extraneous Reducers. Mercuric chloride is added to remove cysteine both preformed and prepared by the action of sulfite on cystine.

"True" cystine is given by the difference between the values found in C and D.

Comment: If both cystine and cysteine are present in the same hydrolysate, it is necessary to correct for the fact that cysteine plus sulfite gives twice the color produced by cystine (*cf.* Lugg, 425; Mirsky and Anson, 460; Schöberl and Rambacher, 568).

There are printer's errors in Lugg's paper (426) on page 2164 lines 13, 33, and 34. B, B, and A should read b, b, and a.

Schöberl and Rambacher (568) use an acetic acid-sodium acetate buffer, *p*H 5.2 (10 parts of 2 M CH_3COONa plus 3 parts of 2 M CH_3-COOH) instead of Lugg's acetate-citrate mixture. The sulfite reduction is run for 20 minutes at 20°C. and the reaction with the color reagent for 30 minuets at 20°. Reagents blanks are prepared. The Zeiss step-photometer, filter 720 mu is used.

Kassell and Brand (356) also use the Zeiss photometer for determining cystine and cysteine according to Lugg's modification of the

Winterstein-Folin reaction. Half the quantities of reagents suggested by Lugg (425) are used. The color development is allowed to proceed for 8 minutes at 25° rather than for 7 minutes as suggested by the original proponent of this procedure.

Bálint (45) in the same year, also adapted the Folin-Lugg method to the Zeiss photometer. He found that Beer's law held over the range 0.16 to 0.75 mg. of cystine, final volume 20 ml., filter 720 mu.

E. The Winterstein-Folin Reaction according to Mirsky and Anson (460)

Principle: Any cysteine present in the protein is oxidized to cystine with peroxide before hydrolysis.

Reagents: Phosphomolybdic Acid (Wu). 100 gm. of $Na_2MoO_4 \cdot 2H_2O$ are dissolved in 450 ml. of water, 15 ml. of 85 per cent H_3PO_4 and 80 ml. of concentrated HCl. The solution is heated under reflux for 8 hours. Any reduced molybdenum is oxidized by warming with a little bromine and the excess Br_2 is driven off by boiling. The solution is diluted to 1 liter.

Method: 1. Hydrolysis. The protein is hydrolyzed with 6 N H_2SO_4 for 15 hours.

2. Cysteine. To 1 to 5 ml. of hydrolysate (0.5 mg. of cysteine), N H_2SO_4 is added to 5 ml., then 16 ml. of concentrated urea (100 gm. of urea in 100 gm. of water), 4 ml. of 3.4 M K_2HPO_4-KH_2PO_4 buffer of pH 6.7–6.8 (1:1 K_2HPO_4-KH_2PO_4), and 1 ml. of either Folin's (236) phospho-18-tungstic acid reagent or Wu's (693) phosphomolybdic acid reagent are introduced. After 5 minutes, the solution is diluted to volume and the color is read.

3. Cystine. A sample of protein is dissolved in 100 ml. of alkali (pH 9.6) and oxidized with 5 ml. of 30 per cent hydrogen peroxide at room temperature for 30 minutes. The protein is removed from solution by precipitation with trichloracetic acid. The hydrolysis and cystine determination are carried out in the same way as given above except 14 ml. of urea solution instead of 16 ml. are used and the cystine is reduced by 1 ml. of 20 per cent Na_2SO_3 (freshly prepared) for 1 minute.

F. Shinohara's Modification of the Winterstein-Folin Reaction (580, 581, 582)

Principle: The acid hydrolysate is extracted two or three times with chloroform to remove mercaptans and other extraneous reducers. Formaldehyde is used to repress color formation of cysteine (*cf.* 48).

Reagents: Bisulfite: 1 M NaHSO₃ is adjusted to pH 5.0 with M NaOH. This solution keeps for 70 days.

Method: 1. Neutralize the hydrolysate with lithium hydroxide to approximately pH 5.2 (brom cresol purple) just before use.

2. *Cysteine.* A. Add to a 50 ml. flask, 10 ml. of 2 M sodium acetate, 3 ml. of 2 M acetic acid, 2 ml. of neutralized, pH 5.2, hydrolysate (0.5 mg. of cysteine), 4 ml. of phospho-18-tungstic acid, and water to 50 ml. Mix and read in 5 to 20 minutes.

B. Repeat A except 1 ml. of 37 per cent HCHO is added to the flask 1 to 2 minutes before the color reagent.

Cysteine $= I_A - I_B$; where B is the color contributed by the non-cysteine reducers and I is the extinction coefficient.

3. *Cystine.* C. Add to a 50 ml. flask, 10 ml. of 2 M sodium acetate, 3 ml. of 2 M acetic acid, 2 ml. of neutralized hydrolysate, 3 ml. of M NaHSO₃, 4 ml. of color reagent, and dilute to volume with water. Read in 15 to 20 minutes.

D. Repeat C except, add 3 ml. of 0.1 M HgCl₂ before the color reagent is introduced.

Cystine $= I_C - I_D$; where D is the color contributed by the non-cystine reducers.

Cystine and cysteine are calculated as indicated by the previous discussion. Remember, cysteine plus sulfite has twice the reducing power of an equal quantity of cystine.

Reactions: Shinohara (582) suggests the following mechanism for Winterstein's reaction

$$2RSH + (H_2O)_3 \cdot P_2O_5 \cdot (WO_3)_{18} \rightarrow$$
$$RSSR + (H_2O)_3 \cdot P_2O_5 \cdot (WO_3)_{17} \cdot WO_2 + H_2O$$
or
$$(H_2O)_3 \cdot P_2O_5 \cdot (WO_3)_{16} \cdot W_2O_5 + H_2O$$
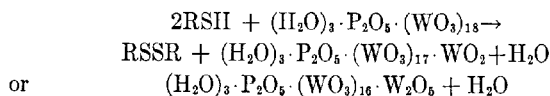
**G. Block and Bolling's Use of the Winterstein-Folin Reaction* (104)

Introduction: The estimation of cystine in an hydrolysate can be carried out with considerable accuracy in 15 or 20 minutes by the procedure given below. Except with protein preparations containing large quantities of carbohydrate, the results obtained by this procedure are equal to those carried out by the more tedious Lugg modifications of the Folin reaction.

Method: 1. Hydrolysis. 50 to 250 mg. of protein are hydrolyzed under reflux with 2 to 5 ml. of 18 per cent HCl for 5 to 7 hours or with an equal mixture of 18 per cent HCl and 90 per cent HCOOH for 18 hours. At the end of the hydrolysis, the solutions are evapo-

rated to a thick syrup in a dish on the steam bath. This removes
some of the excess acid and may convert any cysteine to cystine.
The residues are dissolved in warm water, diluted to volume and
filtered through soft, dry paper. The solutions should react negative
to the nitroprusside test. Decolorize with carbon if necessary.

2. Determination. 2 aliquots (0.4 to 1.2 mg. of cystine) are pipet-
ted into 50 ml. stoppered-graduated cylinders. The solutions are
brought to the 5 ml. mark with water. One ml. of 10 per cent
Li_2SO_4 and 5 ml. of saturated $NaHCO_3$ are now added. After mixing,
2 ml. of Folin's phospho-18-tungstic acid, previously diluted with
an equal volume of water, are added, followed immediately with
either 1 ml. of water (for the blank) or 1 ml. of freshly prepared 10
per cent Na_2SO_3. The solutions are mixed and the flasks are placed
in water at about 30° for 5 to 8 minutes. At the end of this time,
the solutions are diluted to volume with water and read against the
"blank" in a photoelectric colorimeter (photometer). Filter 520
mu. Range 0.1 to 1.2 mg. of cystine.

3. DETERMINATION OF CYSTINE BY IODINE TITRATION

Historical: In 1901, K. A. H. Mörner (463) in his classical paper
on sulfur in proteins pointed out that cysteine could be estimated
quantitatively·by titration with iodine in 10 per cent hydrochloric
acid solution. Starch was used as the indicator.

A. Okuda's Use of the Mörner Reaction (482)

Principle: The cystine in a protein hydrolysate is reduced with
zinc and the resulting cysteine is titrated with standard iodate-
iodide under conditions carefully controlled with respect to acid
concentration, temperature, volume, etc.

Reagents: M/300 KIO_3. 2.14 gm. of KIO_3 are dissolved in 3 liters
of 2 per cent HCl.

Method: 1. Hydrolysis and Reduction. 1 to 10 gm. of protein
are refluxed with 3 to 30 ml. of HCl for 20 hours. The excess acid
is removed by concentration *in vacuo.* The hydrolysate is decolor-
ized with activated carbon and the cystine is reduced with an excess
of Zn dust at room temperature for 30 minutes. The solution is
filtered and diluted to 100 ml.

2. Acid Concentration. An aliquot of the hydrolysate is titrated
to determine the free HCl and then the *free* HCl in the remainder
of the hydrolysate is adjusted to exactly 2 per cent by the addition
of NaOH.

3. Titration of Cysteine. 20 ml. or less of the partly neutralized
hydrolysate are pipetted into an Erlenmeyer flask and diluted to 20
ml. with 2 per cent HCl. 5 ml. of 5 per cent aqueous KI and 5 ml.

of exactly 4 per cent HCl are added and the solution is titrated to
permanent yellow with $M/300$ KIO₃.

The results are compared with a cystine standard treated in
exactly the same way and under the same conditions of temperature,
etc.

Comment: Teruuchi and Okabe (603) add 0.5 ml. of 0.5 per cent
soluble starch in 36 per cent NaCl and titrate to a deep indigo color
which lasts for 1 minute. One half ml. extra of 4 per cent HCl is
added to the Okuda procedure to compensate for the 0.5 ml. of
starch solution.

Lucas and King (423) in a critical study of the Mörner-Okuda
method say that no simple rules can be laid down which will ensure
quantitative results. Temperature, acidity, and concentration of
cysteine and iodine, the rate and order of addition of the reagents
and the presence and concentration of the KI all affect the iodine
consumption in variable ways and to different degrees. They advise
the following: temperature, 0°; acidity, N HCl; KI concentration,
0.5 per cent; and the use of starch as the indicator.

Theoretical results ($2RSH + I_2 \rightarrow RSSR + 2HI$) were not ob-
tained.

B. The Mörner-Okuda Method According to Virtue and Lewis (664)

Principle: Iodine in KI is used instead of KIO₃ + HCl + KI. The
excess I_2 is titrated with standard $Na_2S_2O_3$.

Method: 1. Reduction. The hydrolysate is adjusted to contain
2 per cent of free HCl and the cystine is reduced with zinc dust at
room temperature for 30 minutes.

2. Preparation of Solutions. While the reduction is proceeding,
three Erlenmeyer flasks containing 5 ml. of I_2 in KI (1.27 gm. of
pure I_2 and 2 gm. of KI are dissolved in 400 ml. of water, filtered
and diluted to 1 liter) are placed in a freezing mixture. The reagent
blank is determined on one of the solutions by adding 15 ml. of
2 per cent HCl and titrating with 0.02 N $Na_2S_2O_3$. 0.5 ml. of 2 per
cent soluble starch in saturated NaCl are used as the indicator.

3. Preliminary titration. 15 ml. of the hydrolysate containing
cysteine and 0.5 ml. of starch solution are added to the frozen io-
dine. Then 0.02 N $Na_2S_2O_3$ is added from a burette at such a rate
that the blue color which develops as the iodine is liberated by melt-
ing is immediately destroyed.

4. Final Titration. To the frozen I_2, add 0.05 ml. *less* of the
$Na_2S_2O_3$ solution than required by the "Preliminary Titration,"
then add 15 ml. of the hydrolysate and shake to remove the blue

color as fast as it appears. The titration should be completed as the last of the frozen I_2 solution melts.

C. Lavine's Modification of the Mörner-Okuda Reaction (406)

Principle: Cysteine is quantitatively oxidized to cystine by molar HI at room temperature.

Method: A cysteine containing solution is adjusted to M HI by the addition of KI and HCl. 10 ml. aliquots are removed and titrated with 0.025 N $Na_2S_2O_3$ until the yellow color has disappeared.

The end point is checked by the addition of 5 to 10 ml. of 0.1 per cent starch in 0.1 per cent aqueous salicylic acid. If the solution is blue, 0.02 ml. portions of standard $Na_2S_2O_3$ are added in 10 second intervals with swirling until the solution becomes colorless. The end point is again verified by the addition of several drops of I_2 whereupon the brownish violet color should return.

4. SULLIVAN'S REACTION (595, 596, 597, 598, 602, etc.)

Historical: In 1926, M. X. Sullivan showed that, of all the amino acids normally occurring in a protein hydrolysate, only cysteine gave a distinctive red color in alkaline solution with 1,2,naphthoquinone-4-sodium sulfonate in the presence of $Na_2S_2O_4$.

A. Sullivan's Original Method, 1926 (595)

Reagents: Preparation of β-Naphthoquinone-Sodium Sulfonate (Folin, 230). 1. Dissolve 100 gm. of β-naphthol (resublimed) in 300 ml. of 10 per cent NaOH.

2. In a 4 liter beaker, dissolve 50 to 55 gm. of $NaNO_2$ in 600 ml. of water.

3. Add β-naphthol solution to nitrite.

4. Add 300 gm. of crushed ice to mixture.

5. Add 200 ml. of cold 10 per cent H_2SO_4 with vigorous stirring. Continue to stir 1 to 2 minutes after all the acid has been introduced. Repeat above until a total of 800 ml. has been added and then until the pH is permanently acid to Congo red paper. Stand 1 hour.

6. Remove the precipitate and wash it with 1 liter of cold water.

7. Transfer the precipitate to a large evaporating dish and sprinkle over it, 100 gm. of $NaHSO_3$ and 50 gm. of Na_2SO_3. Stir until liquefaction. Filter on a small Buchner funnel to remove tar. Wash the residue with a little water.

8. Transfer the liquid at once into a 5 liter colored flask (or

one covered with dark paper) which contains 2000 ml. of water and 500 ml. of concentrated HCl. Cover same and let stand in the dark for 36 hours. Filter and wash with 2 liters of cold water.

9. Place the precipitate in a beaker and cover the precipitate with 100 gm. of $NaNO_3$. Add a luke warm mixture of 100 ml. of concentrated HNO_3 and 350 ml. of H_2O. N_2O_4 fumes appear. After 10 minutes stir thoroughly for 2 or 3 minutes. Stand 20 to 30 minutes longer. If no reaction takes place on adding the dilute HNO_3, then add 1 to 5 ml. of concentrated acid. Filter the precipitate and wash with 1 liter of 10 per cent NaCl.

10. Place the moist precipitate in a large porcelain dish and add 200 gm. of powdered borax and 450 ml. of water. Mix until almost all the quinone has dissolved. Filter.

11. To the quinone solution, add a cooled mixture of 850 ml. of 95 per cent ethanol and 150 ml. of concentrated HCl to which a few drops of bromine has just been added. Stir vigorously. Stand 5 minutes. Filter the quinone and wash with 700 to 800 ml. of 10 per cent NaCl.

12. Recrystallize the quinone from borax as above except wash with 300 to 400 ml. of alcohol followed by 200 ml. of ether. Yield 75 to 90 gm.

Tests for Purity. 1. A fresh 1 per cent solution of the quinone should have less color than 0.5 N $K_2Cr_2O_7$.

2. Dilute 2 ml. of a 1 per cent solution to 25 ml. Add 1 ml. of 50 per cent acetic acid and then 1 ml. of 15 per cent $Na_2S_2O_3$. Solution should become colorless in a few seconds.

Method: 1. *Cysteine.* To 5 ml. of cysteine solution (0.4 mg.) add 1 ml. of 0.5 per cent, 1,2,naphthoquinone-4-sulfonate. Mix. Then add 5 ml. of 10 per cent Na_2SO_3 in 0.5 N NaOH and 1 ml. of 5 per cent NaCN. Stand 10 to 20 minutes. Remove interfering colors with 1 to 2 ml. of 2 per cent $Na_2S_2O_4$ in 0.5 N NaOH.

2. *Cystine.* "Reduce" 0.4 mg. of cystine in 5 ml. of solution with 1 ml. of 5 per cent NaCN at room temperature for 10 minutes. Continue as above.

Comment: The high degree of specificity of this method indicated great value. However, many investigators reported difficulties in applying the method as originally described.

Prunty (530) reduced the cystine in 0.1 N HCl with a few milligrams of Zn dust. This procedure also removed a great deal of interfering color. Pollard and Chibnall (524) found that Prunty's modification of the Sullivan method gave 90 to 95 per cent of the cystine in grass proteins. They made some further modifications including the use of 1 ml. of 1 per cent naphthoquinone rather than the 1 ml. of 0.5 per cent suggested by Sullivan.

Krijgsman and Bouman (391) used the Zeiss step-photometer, filter 57 *i.e.* 570 mu for cystine in blood by the Sullivan method.

Lugg (427) avoided the effects of varying quantities of amino acid on the color development by flooding the solution with glycine. He also carefully controlled the pH at which the reaction was carried out. Ferrous ions, zinc, and other metals were shown to interfere.

Bushill, Lampitt, and Baker (136), employing the Zeiss step-photometer, filter 530 mu, found that the original Sullivan method had an error of ± 25 per cent, the Lugg procedure one of ± 8 per cent provided that a rapid stream of purified nitrogen was passed through the flask for 30 seconds immediately before and after the addition of the sodium hyposulfite reagent. Bushill *et al.* (136) suggest the addition of varying quantities of cystine to the protein hydrolysate and plotting the extinction coefficients. The line is then extended to no addition and the point where the line cuts the abscissa is a measure of the cystine present in the hydrolysate.

Andrews and Andrews (31) criticize Lugg's modification of the Sullivan method because the resulting colors are too weak and the procedure is too complex. Their change in the Sullivan method consists in quadrupling the quantity of naphthoquinone added and in allowing the solutions to stand in the dark for 30 minutes before the addition of $Na_2S_2O_4$. Andrews and Andrews(31) wait exactly 20 seconds after the addition of the color reagent before adding the Na_2SO_3 solution.

Kuhn and Desnuelle (393) advise the use of a 470 mu filter in Lugg's modification of the Sullivan method.

B. Rossouw and Wilken-Jorden's Use of the Sullivan Reaction (558)

Principle: Cystine is quantitatively precipitated by cuprous chloride at pH 4.5.

Reagents: 500 mg. of Cu_2Cl_2 are shaken with 1 per cent HCl to remove the $CuCl_2$. The white residue of Cu_2Cl_2 is then dissolved in the least quantity of 25 per cent KCl in 0.2 per cent HCl. It is prepared fresh each time.

Method: 1. Precipitation of Cysteine as the Copper Mercaptide. An aliquot of the hydrolysate containing 2 mg. of cystine is pipetted into a 50 ml. centrifuge tube and 2.5 ml. of glacial acetic acid are added. Then sufficient 10 per cent KOH is introduced to bring the reaction to pH 4.5 (brom cresol green). Any humin precipitate is removed and washed with acetate buffer, pH 4.5. After dilution to 40 ml., 5 to 10 drops of the Cu_2Cl_2 reagent are added. After 5 to

60 minutes, the flocculated precipitate is centrifuged and washed with alcohol.

2. Liberation of Cystine from Copper Salt. The precipitate is dissolved in 5 ml. of 1 per cent HCl and the solution is transferred to a 25 ml. graduated cylinder by the aid of water. The following are added in order with mixing, 2.5 ml. of 5 per cent acetic acid, 1 ml. of 10 per cent potassium thiocyanate, and sufficient pyridine to bring the pH to 4.5. The solution is diluted to 25 ml. with water. The copper-pyridine-thiocyanate precipitate is removed.

3. Determination of Cystine. The Sullivan reaction on a suitable aliquot (5 ml.) is used after adding 1 ml. of 10 per cent KOH to overcome buffering.

Comment: Rossouw and Wilken-Jorden (558) point out that if too much cuprous chloride has been added or the concentration of KCl is too high, the copper mercaptide will not all dissolve in 5 ml. of 1 per cent HCl. In such cases, a little Zn dust should be added to convert Cu_2Cl_2 to $ZnCl_2$ and free Cu. After decomposing with KCNS and pyridine, the solution should be aerated or allowed to stand until all the cysteine is oxidized to cystine before applying the Sullivan reaction.

Zittle and O'Dell (698) use cuprous oxide in dilute sodium acetate solution to precipitate the cystine instead of cuprous chloride as recommended by Rossouw and Wilken-Jorden.

C. The Sullivan and Hess 1937 Modification (598)

Principle: The rate of hydrolysis of the protein is greatly accelerated by the introduction of titanous chloride. All cystine formed during the hydrolysis is reduced to cysteine.

Reagents: 20 per cent $TiCl_3$ preserved over Zn dust.

Method: 1. Hydrolysis. Hydrolyze 1 gm. of protein with 5 ml. of 20 per cent HCl and 1 ml. of 20 per cent $TiCl_3$ under reflux for 1 to 2 hours. Temperature of oil bath 125°. Neutralize the hydrolysate with 5 N NaOH, dropwise, to about pH 6. Filter and wash the $Ti(OH)_3$ with 5 ml. of water. Adjust the filtrate to pH 3.5 with HCl and dilute to 35 ml. with 0.1 N HCl.

2. Determination. A. To 5 ml. of filtrate, add 1 ml. of 0.5 per cent naphthoquinone, shake 10 seconds. Add 5 ml. of 10 per cent Na_2SO_3 in 0.5 N NaOH and 1 ml. of 1 per cent NaCN in 0.8 N NaOH, stand 30 minutes. Then add 1 ml. of 2 per cent $Na_2S_2O_4$ in 0.5 N NaOH. Read against *cystine* standard prepared in the same way.

B. The cysteine of the hydrolysate can be converted to cystine by aeration. In this case, 5 ml. of the hydrolysate, cysteine-free, is

treated with 2 ml. of 5 per cent NaCN in n NaOH for 10 minutes before proceeding as in A. Read against a *cystine* standard treated in the same way.

Comment: Sullivan and Hess (598) suggest Carbex E to decolorize the hydrolysates if necessary.

Lugg (431) prevented humin formation and reduced the destruction of cystine by carrying out the hydrolysis with 57 per cent HI under nitrogen. Cystine was determined against a cystine or cysteine standard treated in the same way. A solution of gelatin hydrolyzed with hydriodic acid was used as the reagent blank. Lugg reports random errors of 3 to 5 per cent and a hydrolytic loss of 2 per cent.

D. The 1942 Sullivan-Hess-Howard Procedure (602)

Principle: The use of both nascent hydrogen ($Na \cdot Hg$) and sodium cyanide to reduce the cystine in separate portions of the same solution permits the estimation of both cystine and cysteine in mixtures.

1. $RSSR + H_2(Na \cdot Hg) \rightarrow 2RSH$ (Reduction).

2. $RSSR + NaCN \rightarrow RSNa + RSCN$ (Double Decomposition).

Method: A. Cyanide "Reduction." To 5 ml. of solution (1 mg. of cystine), add 2 ml. of freshly prepared 5 per cent NaCN, mix and stand for 10 minutes. Add 1 ml. of 1 per cent sodium 1,2,-naphthoquinone-4-sulfonate, shake for 10 seconds, add 5 ml. of 10 per cent Na_2SO_3 in 0.5 n NaOH, mix and stand for 30 minutes. Then add 1 ml. of 5 n NaOH and 1 ml. of 2 per cent $Na_2S_2O_4$ (hyposulfite, dithionite) in 0.5 n NaOH. Read against a cystine standard prepared in the same way.

B. Sodium Amalgam Reduction. Reduce 2 mg. of cystine in 10 ml. of 0.1 n HCl with 1 ml. of 0.2 per cent sodium amalgam for 1 hour at room temperature. Shake occasionally. Remove 5 ml. of the supernatant solution and determine its "cystine" content as given above.

$$Cysteine = B\text{-}A$$
$$Cystine = B\text{-}cysteine$$

Comment: It is obvious from equations 1 and 2 (Principle) that equal weights of cystine and cysteine treated by Na amalgam would give the same amount of color, which would be twice that given by an equal quantity of cystine treated with cyanide.

5. PRECIPITATION OF CYSTEINE AS THE CUPROUS MERCAPTIDE

Historical: In 1929, F. G. Hopkins made the interesting observation that thiol compounds (reduced glutathione) are quantitatively precipitated from solution in dilute sulfuric acid (0.5 n to

1.0 N) by the addition of a slight excess of cuprous oxide. This finding was the basis for the following methods for the estimation of cystine.

A. The Procedure of Vickery and White (652)

Principle: Cysteine is formed during the hydrolysis by refluxing in the presence of tin. Cysteine and any other sulfhydryl compounds present are then precipitated by the addition of Cu_2O.

Reagents: Cuprous Oxide (Zittle and O'Dell, 698). 7 gm. of $CuSO_4 \cdot 5\ H_2O$ in 100 ml. of water are added to 100 ml. of a solution made from 170 gm. of sodium tartrate dihydrate, 520 ml. of water, and 80 ml. of saturated NaOH. The Fehling's Solution is heated to boiling and 1 gm. of glucose in 200 ml. of water is added; after boiling for 3 minutes, the precipitate of Cu_2O is washed by decantation.

Method: 1. Hydrolysis. 2 gm. of protein (20 mg. of cystine) are hydrolyzed by boiling with 20 ml. of 8 N H_2SO_4 for 24 hours. After the protein has dissolved, 2 to 3 gm. of coarse tin are added.

2. Precipitation of Cysteine Cuprous Mercaptide. At the end of the hydrolysis the precipitate is removed, and the filtrate is diluted to 100 ml. The solution is warmed to 40 to 45° and an aqueous suspension of Cu_2O is added in 200 mg. quantities until an excess is present. This is indicated by the change of color of the precipitate from grey to pink or red. The solution should be stirred rapidly during the addition of the cuprous oxide. The solution is then cooled to 10°C. and 10 N NaOH is added drop by drop to *p*H 4 to 5 (red to Congo paper). The suspension is cooled over night.

3. Decomposition of Cuprous Mercaptide. The precipitate is removed by centrifugation and washed thoroughly with water to remove the H_2SO_4. The washed precipitate is then suspended in 400 ml. of water containing 1 to 2 ml. of HCl and the copper salt is decomposed with H_2S. The Cu_2S precipitate is washed with dilute HCl. The filtrate and washings are concentrated to 150 ml. and a slight excess of barium hydroxide is added to remove any H_2SO_4. The $BaSO_4$ is removed and washed with dilute HCl.

4. Determination of Cystine. Cystine is estimated either from the nitrogen or sulfur in the filtrate. The oxidizing mixture of Waelsch and Klepetar (668), consisting of 50 gm. of $Cu(NO_3)_2$, 10 gm. of NH_4NO_3 and 25 gm. of NaCl in 100 ml. of water plus saturated Na_2CO_3 until alkaline to litmus, is advised if S is to be determined.

Comment: The authors claim agreement within 10 per cent between their method and other procedures in the literature.

B. Graff's Micro Modification of the
Hopkins-Vickery Procedure (264)

Reagents: Citrate-Acetate Buffer. 20 ml. of glacial acetic acid, 12 gm. of sodium citrate and 15 gm. of citric acid are dissolved in 200 ml. of water.

Method: 1. Hydrolysis and Reduction. Sufficient protein to contain 2 to 5 mg. of cystine is hydrolyzed with 18 per cent HCl. The excess acid is removed by distillation *in vacuo.* The residue is dissolved in 10 ml. of water and the cystine is reduced with 100 mg. of zinc dust by heating under reflux for 1 hour.

2. Precipitation. 8 N sodium acetate is added to bring the solution to pH 5. After 15 minutes, the precipitate is filtered and washed with hot water. Volume = 30 to 35 ml. Glacial acetic acid is added to the filtrate to pH 4, the solution is heated to boiling and a Cu_2O suspension is added dropwise during mechanical stirring, until the precipitate has acquired an orange or dull-red color. The copper salt is centrifuged and washed 4 times with dilute (1:10) citrate-acetate buffer.

3. Determination of Cystine. The precipitate is transferred to a suitable vessel and oxidized with 1 ml. of ignition mixture (10 gm. of K_2CO_3, 20 gm. of KNO_3, and 10 gm. of $KClO_3$ dissolved in the least amount of water). The mixture is evaporated to dryness and then held at red heat for 5 minutes. Sulfate is determined gravimetrically with Ba^{++} as usual.

Comment: Although Vickery (652) and Graff (264) believed that cystine could be determined with equal accuracy from either the total nitrogen or sulfur of the copper precipitate, Schultz and Vars (572) advise against using the nitrogen values and Lucas and Beveridge (424) say "Certainly little trust should be placed in the accuracy of cysteine determinations based upon the nitrogen content of mercaptide precipitates."

Zittle and O'Dell (698) found, in agreement with Rossouw and Wilken-Jorden (558), that reduction of cystine to cysteine before precipitation with cuprous oxide is not necessary. They advise the use of the Graff or Rossouw adaptations of Hopkins' Cu_4O precipitation.

6. CONDENSATION WITH DIMETHYL-p-PHENYLENEDIAMINE (FLEMING, 225)

Historical: One of the standard procedures for the determination of hydrogen sulfide consists in condensing it with dimethyl-p-phenylenediamine in the presence of zinc to yield methylene blue. In 1930, R. Fleming showed that cysteine would also yield a blue

colored compound when warmed with dimethyl-p-phenylenediam-
ine in the presence of $FeCl_3$.

A. Toyoda's Adaptation of the Fleming Reaction (618)

Principle: Cystine is reduced by zinc in HCl and the resulting
cysteine is warmed with dimethyl-p-phenylenediamine in the pres-
ence of ferric ions.

Reagents: Dye: 500 mg. of dimethyl-p-phenylenediamine hy-
drochloride are dissolved in a cooled mixture of 100 ml. of water
and 50 ml. of concentrated H_2SO_4. Water is added to 1 liter.

Ferric Alum: 25 gm. of $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot 24 H_2O$ are dis-
solved in 100 ml. of water and 5 ml. of concentrated H_2SO_4. The
solution is diluted to 200 ml.

Water: Prepare S free water if necessary by distilling from
$KMnO_4$.

Method: 1 ml. of cystine solution (0.05 to 0.25 mg. of cystine) is
placed in a graduated cylinder with 50 mg. of Zn dust and exactly
0.50 ml. of N HCl. Immediately add 7.5 ml. of dimethyl-p-phe-
nylenediamine reagent followed by 0.5 ml. of ferric alum. The
solution is diluted to volume (25 ml.), stoppered and read after 12
hours against a standard prepared in the same way.

Comment: It is necessary to have the concentration of the acid
the same in both unknown and standard.

*B. Vassel's Use of the Fleming Reaction (641)

Reagents: Dye: Dissolve 35 mg. of dimethyl-p-phenylenediamine
hydrochloride (Eastman #492) in 100 ml. of exactly 6 N H_2SO_4.
Prepare fresh every two weeks. Keep in refrigerator.

Ferric Alum: Dissolve 20 gm. of $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot 24 H_2O$
in 100 ml. of N H_2SO_4.

Method: 1. Hydrolysis. Hydrolyze 25 to 100 mg. of protein under
reflux for 18 hours with 2 ml. of a mixture prepared from 58 ml. of
concentrated HCl and 63 ml. of 85 to 90 per cent HCOOH. Dilute
the hydrolysate to 10 ml. with 5 N Hcl.

2. Estimation of Cysteine. Place 165 mg. of Zn dust, 3 ml. of
dye solution and 2 ml. of ferric alum in a test tube and stand at
room temperature for 10 minutes. Place the tube in boiling water
to dissolve the zinc (15 to 35 minutes). Cool and add 1 ml. of hy-
drolysate (0.05 to 0.20 mg. of cysteine), 3 ml. of ferric alum, mix and
heat in boiling water for 45 minutes. Cool, dilute to 25 ml., read
against a standard prepared with the same acid concentrations.
Light filter 575 to 580 mu.

3. Estimation of Cystine. Pipette 1.0 ml. of unknown (0.05 to
0.20 mg. of cystine) into a test tube, then add 3 ml. of dye, and 165

mg. of zinc dust. Stand 2 to 4 minutes and add 2 ml. of ferric alum,
mix from time to time. Stand at room temperature for 45 minutes.
Add 3 ml. more of ferric alum and place the stoppered tubes in a
boiling water for 45 minutes, to dissolve all the zinc. Cool, dilute to
25 ml. with water. Read using filter 575 to 580 mu against a blank
of reagents.

Comment: The amount of color developed is subject to change
with variations in the strength and quantities of acid. It is most
important, therefore, to have all conditions carefully controlled.

7. Gasometric Estimation of Cystine and Cysteine (Baernstein, 39)

Principle: Cysteine reacts with iodine, the excess I_2 is then de-
termined gasometrically by the production of nitrogen from hy-
drazine (*cf.* Van Slyke and Hawkins, 635).

Comment: This procedure will not be described as it does not ap-
pear to have been used except by Baernstein (39, 41). The pro-
cedure has been criticized by Hess (290) as giving erroneously high
results.

8. Polarographic Determination of Cystine (Brdicka, 130, 131)

Historical: In 1933, Brdicka (130) found that the dropping mer-
cury cathode polarograph could be used to detect small quantities
of proteins if cobalt salts were present. The reactive group in the
proteins was shown to be the disulfide of cystine. This finding sug-
gested a method for the determination of cystine and other disul-
fides in very small quantities of protein hydrolysates (131).

Although numerous investigators, including Brdicka and Sulli-
van and Hess, have reported successful results with the polaro-
graph only the procedure of Stern, Beach and Macy will be given
(590).

A. The Use of Brdicka's Method by Stern, Beach, and Macy (590)

Principle: Cystine exerts a catalytic effect on the separation
of hydrogen from water by means of the cobalt complex compound
of cysteine.

Apparatus: The standard dropping mercury polarograph devel-
oped by Heyrovsky is used. Heyrovsky showed that under the
proper conditions, when the dropping mercury electrode is em-
ployed, an ion in solution makes its presence known by a current
increase which takes place at a definite voltage; and second, there
is a definite relationship between the amount of the current in-

crease at that voltage and the concentration of the ion causing the increase. Thus, both qualitative and quantitative analyses based on voltage and current measurements, respectively, can be made at one time.

Determination: 0.5 ml. of a protein hydrolysate are dissolved in 24.5 ml. of a solution of 0.1 N NH_4Cl; 0.1 N NH_4OH and 10^{-2} M $CoCl_2$. The wave height is determined and calculated as zero. Then increasing quantities of cystine are added to 0.5 ml. aliquots of the hydrolysate and the wave heights are found under the same conditions. The above is repeated with 1.0 ml., 1.5 ml., and 2.0 ml., portions of the hydrolysate. The quantity of cystine in the unknown is calculated from the curves so obtained.

Comment: It is necessary to keep the voltage, depth of the mercury, temperature, rate of Hg drops, etc. constant.

When a series of homologous proteins, having approximately the same composition with respect to amino acids other than cystine, are being analyzed it is not necessary to prepare new calibration curves with each hydrolysate. However, a calibration curve must be made for each class of protein.

It is claimed that a determination can be carried out in 90 minutes.

9. THE NITROPRUSSIDE REACTION

Historical: Mörner (463) appears to have been one of the first to suggest the use of sodium nitroprusside in dilute alkali as a quantitative method for the estimation of cysteine. The specificity of the nitroprusside reaction for cysteine among the naturally occurring amino acids was confirmed by Arnold (35) who advised the use of NH_4OH in preference to NaOH and KOH as the alkalinizing agent. The addition of acetic acid promptly decolorizes the solution.

A. Shinohara and Kilpatrick's Use of the Nitroprusside Reaction (579)

Method: To 5 ml. of unknown solution containing cysteine, add 2 ml. of 0.2 M zinc acetate, 2 ml. of M NH_4OH, and 0.5 ml. of 5 per cent sodium nitroprusside in the above order. Mix and compare with a cysteine standard treated in the same way and at the same temperature.

B. Krishnaswamy's Modification of the Mörner Test (392)

Method: Add 1 ml. of HCl to a solution of 0.8 to 1.5 mg. of cystine, dilute to 5 ml. and add, with shaking 2 ml. of 5 per cent aqueous NaCN and 1 ml. of 20 per cent Na_2SO_3. Stand 1 minute, add 10 ml. of 0.5 N NH_4OH, wait 5 minutes, add 0.2 ml. of 0.3 M $ZnSO_4$ and 1 ml. of freshly prepared 5 per cent $Na_2Fe(CN)_5NO \cdot 2 H_2O$, mix and read immediately. The color is stable for 6 to 7 minutes at 29°.

10. ESTIMATION OF CYSTEINE BY ITS REDUCING ACTION ON SULFUR

Historical: Although the production of H_2S by S from tissues had been observed by various French workers in the latter part of the 19th Century; it was not until 1907 that Heffter (285) showed that the reduction of sulfur by proteins and -SH compounds was stoichiometric and not catalytic.

A. The Method of Guthrie and Allerton (275)

Principle: Free -SH groups are determined by their reducing action on colloidal S to yield H_2S.

Reagents: Colloidal S. Pipette rapidly 20 ml. of hot saturated absolute alcohol solution of sulfur into 50 ml. of boiling water. Evaporate to 20 ml. and then dilute to 25 ml. Prepare fresh each day.

Method: 1. Production of H_2S. To 10 ml. of unknown (protein or -SH containing compounds) add 10 ml. of M/15 phosphate buffer, *p*H 7.0 ± 0.1, a few drops of cetyl alcohol and 1 ml. of colloidal S. Place in a 30° bath and aerate the H_2S into 25 ml. of 2 per cent zinc acetate solution. Continue the aeration for 4 hours using O_2-free nitrogen which is bubbled through a flask containing some aqueous HCN.

2. Determination of H_2S. A. Iodometrically. Add 10 ml. of N/1000 KIO_3, 2 ml. of 1 per cent KI, 10 ml. of 1:5 HCl, and 1 ml. of soluble starch solution to the zinc acetate solution. Titrate the excess I_2 with N/1000 $Na_2S_2O_3$.

B. Colorimetrically. Add 5 ml. of 0.1 per cent dimethyl-p-phenylenediamine hydrochloride in 20 per cent HCl and 5 ml. of M/50 $FeCl_3$ in 1:9 HCl to the zinc acetate solution. Stand over night, dilute to 50 ml. and read the amount of methylene blue formed.

Comment: The procedure of Guthrie and Allerton (275) appears to give considerable promise as a specific method for the determination of cystine and cysteine in proteins without the necessity of complete hydrolysis.

11. MISCELLANEOUS METHODS FOR THE DIRECT DETERMINATION OF CYSTEINE

A. The Iodoacetate Reaction (Rosner, 554)

Principle: The sulfhydryl groups of proteins react with iodoacetic acid to liberate HI. The quantity of HI liberated is determined.

$$RSH + ICH_2COOH \rightarrow RSCH_2COOH + HI$$
$$2HI + H_2O_2 \rightarrow I_2 + 2 H_2O$$
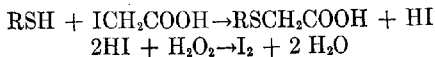
Method: To 3.5 ml. of solution, containing a known quantity of soluble protein, add 1.5 ml. of M phosphate buffer, *pH* 7.3, and 5 ml.

of 0.1 N ICH_2COOK. Stand at room temperature for varying lengths of time, 40 minutes to 6 hours. Then add 0.25 ml. of concentrated H_2SO_4, 0.25 ml. of CCl_3COOH (100 gm. plus water to 100 ml.), filter and add 0.1 ml. of 3 per cent H_2O_2. Read the iodine colorimetrically. Extrapolate to zero time.

$$\text{Per cent of Cysteine} = \text{mg. of Iodine} \times \frac{121.12}{126.93} \times \frac{100}{\text{mg. of protein}}.$$

B. The Iodosobenzoic Acid Method (Hellerman, 288)

Principle: o-Iodosobenzoate reacts with sulfhydryl groups.

$$2\ RSH + OIC_6H_4COOK \rightarrow RSSR + IC_6H_4COOK + H_2O$$
$$OIC_6H_4COOH + 2HI \rightarrow IC_6H_4COOH + I_2 + H_2O$$
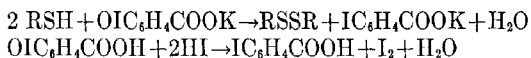
Reagents: Purification of o-iodosobenzoic acid. The free acid is dissolved in the least excess of KOH and then precipitated with CO_2, washed with water and dried over P_2O_5.

Phosphate Buffer, *p*H 7. Dissolve 117.7 gm. of K_2HPO_4 and 44.1 gm. of KH_2PO_4 in water and dilute the solution to 1 liter.

Method: Add 5 ml. of M phosphate buffer, *p*H 7, and 10.0 ml. of 0.02 N o-iodosobenzoate to 10 ml. of the unknown -SH compound. Wait 30 seconds and add a freshly prepared mixture of 500 to 1000 mg. of KI in 1.5 ml. of water and 5 ml. of N HCl. Titrate the liberated iodine at once with standard Na_2SO_3 using starch as the indicator.

Comment: Rosner's (554) and Hellerman's (288) methods for the direct determination of sulfhydryl groups seems to be better than titration with porphyrindin as the latter dye has been reported to be oxidized by tyrosine at *p*H 7.2 (124).

12. DIRECT OXIDATION OF CYSTINE TO SULFATE (CALLAN AND TOENNIES, 137A)

Principle: Cystine is quantitatively oxidized to inorganic sulfate by warming with an excess of alkaline permanganate in aqueous solution. Methionine does not form any sulfate under these conditions.

Method: 1 gm. of wool, 10 gm. of $KMnO_4$, and 160 millimols of NaOH are added to 150 ml. of water. The mixture is digested on the steam bath for 48 hours. The excess $KMnO_4$ is destroyed with methanol, and after acidification, the MnO_2 is filtered off. SiO_2 is also removed. The sulfate is then precipitated as usual from the boiling solution with $BaCl_2$.

Comment: This method appears to be of special value when estimations of cystine are to be made in protein preparations heavily contaminated with carbohydrate.

CHAPTER III

PART III

THE DETERMINATION OF METHIONINE AND HOMOCYSTINE

1. DETERMINATION OF METHYL GROUPS IN PROTEINS

Historical: Barger and Coyne (50) were the first to point out that methionine could be determined by demethylation with hydriodic acid and estimation of the resulting methyl iodide. This procedure was used by Baernstein (40) to determine the quantity of methionine in proteins.

A. Baernstein's Original Volatile Iodide Method (40)

Principle: The protein is hydrolyzed and demethylated simultaneously by boiling under reflux with concentrated HI.

$$CH_3SCH_2CH_2CHNH_2COOH+HI \rightarrow HSCH_2CH_2CHNH_2COOH$$
$$+CH_3I$$
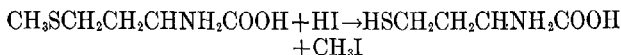
Reagents: Silver Nitrate. 8 gm. of $AgNO_3$ are boiled under reflux for 30 minutes with 500 ml. of absolute alcohol. The solution is allowed to stand for 2 days, filtered, standardized and kept in the dark.

Purified CO_2 or N_2. The gas is passed through two washers, one of which contains concentrated H_2SO_4, the other contains dilute $AgNO_3$.

Method: 1. Hydrolysis and Demethylation. 500 mg. of protein are placed in a 100 ml. digestion flask, with a quartz pebble, and 10 ml. of redistilled HI (sp. gr. 1.7) containing 1 per cent KH_2PO_2 are added. The flask is attached to a reflux condenser and is provided with a side-arm to permit the introduction of a stream of purified carbon dioxide or nitrogen *over* the boiling solution. The flow of gas is regulated so that the bubbles can be counted. The methyl iodide is aerated into a series of washers, the first containing 10 ml. of 20 per cent $CdSO_4$ in dilute H_2SO_4 and 1 ml. of a suspension of red P, the second and third each contain 10 ml. of standard $AgNO_3$ in absolute alcohol. The hydrolysis is continued 6 to 8 hours or longer.

2. Determination of Methyl Iodide. The contents of washers 2 and 3 are rinsed into a beaker and evaporated to 10 ml. This solution is then diluted to 50 ml. in a volumetric flask and filtered. 5 ml.

aliquots of the clear filtrate are titrated with 0.02 N KSCN after the addition of 2 ml. of HNO_3 and 2 ml. of saturated ferric alum. A blank without the protein is run on all the reagents.

Comment: The use of concentrated HI to hydrolyze proteins was introduced by Kossel and Kutscher in 1901. As glycerol, ethanol, methanol, ether, and numerous other substances yield CH_3I and C_2H_5I under these conditions, they must be absent.

The results for methionine by this procedure tend to be "high."

Bailey (43) observed that a small amount of H_2S passed over into the standard $AgNO_3$ washers and has suggested that the precipitate of silver iodide and sulfide be digested with dilute HNO_3. The $AgNO_3$, regenerated from the Ag_2S is determined and the appropriate correction applied.

2. Determination of Methionine from Homocysteine

A. *Baernstein's Volatile Iodide and Homocysteine Titration Method* (42)

Principle: Cysteine and homocysteine thiolactone are formed during the hydrolysis of a protein with HI. The cysteine can be determined by oxidation with iodine to cystine. The ring of the homocysteine thiolactone is then opened with alkali (NH_4OH) and the resulting homocysteine in turn is titrated with I_2.

Apparatus: Similar to that used in A above.

Reagents: Na Tetrathionate. Prepare fresh from 0.1 N $NaIO_3$ +0.1 N $Na_2S_2O_3$ +a little KI and HCl.

Method: 1. Hydrolysis. 500 mg. of protein are boiled under reflux with 10 ml. of 57 per cent HI containing 1 per cent KH_2PO_4 for 6 hours. The CH_3I formed during the reaction is aerated by a slow stream of purified N_2 (*cf.* 1. above) into an adsorption train. Washer No. 1 contains 10 ml. of 20 per cent $CdCl_2$ with 20 per cent $BaCl_2$. No. 2 contains 10 ml. of saturated $HgCl_2$. Nos. 3 and 4 contain 10 ml. each of 10 per cent CH_3COOK in glacial CH_3COOH plus 6 drops of bromine. The stream of gas is not started until the free iodine formed at first has been destroyed by the KH_2PO_2. At the end of the hydrolysis, while continuing the stream of N_2, the flask is removed from the condenser and the digest is concentrated on a free flame to 3 ml. Do not burn.

2. Analysis of Digest. A. Cysteine. 2 or 3 crystals of KH_2PO_2 are added to the digest which is boiled for one minute to destroy the I_2. The solution is rinsed into a 25 ml. volumetric flask with 4 per cent HCl. After cooling, it is diluted to 25 ml. A 10 ml. aliquot of this solution is placed in a 50 ml. Erlenmeyer flask, which is deaerated at the pump. 2 ml. of 0.02 N KIO_3 are added for

each 4 mg. of cystine present. Then 1 ml. of soluble starch is added and the iodine is titrated with 0.02 N $Na_2S_2O_3$. A blank is run.

1 ml. of 0.02 N $Na_2S_2O_3 \approx 2.40$ mg. of Cystine.

B. Homocysteine. 2 ml. of $Na_2S_4O_6$ are added to the digest which is then deaerated. The connection between the pump and the flask is made through a 3 way stop-cock and a rubber or ground glass stopper. When the flask has been evacuated, 3.0 ml. of NH_4OH are added from a burette and the flask is again evacuated. After 15 minutes, the solution is acidified with 10 ml. of 10 per cent HCl and the homocysteine is titrated with 0.02 N KIO_3 under nitrogen. A reagent blank is run.

1 ml. of 0.02 N $KIO_3 \approx 2.98$ mg. of Methionine.

3. Determination of Volatile Iodide. Washers Nos. 3 and 4 are rinsed into a 100 ml. volumetric flask which already contains 5 ml. of 25 per cent sodium acetate. A little HCOOH (sp.gr. 1.20) is added to destroy the Br_2. The solution is mixed and diluted to volume. 25 ml. aliquots are removed. One to 2 gm. of KI and a few ml. of 10 per cent H_2SO_4 or HCl are added and the I_2 is titrated with 0.02 N $Na_2S_2O_3$ using starch as the indicator towards the end of the reaction.

1 ml. of 0.02 N $Na_2S_2O_3 \approx 0.496$ mg. of Methionine.

Comments: The protein should be extracted with petroleum ether and dried at 105° to remove any traces of alcohol, ether, etc. (42) or first hydrolyzed with HCl, evaporated to dryness and digested with HI (43). Bailey (43) suggests a correction factor of 1.05 for methionine by the volatile iodide method.

Kassell (357) has suggested the following changes in Baernstein's procedure: a. The titrations are carried out on the whole digest and all solutions which contain sulfhydryl groups are kept under nitrogen. b. Homocysteine is determined by adding 2 to 3 drops of brom thymol blue indicator, 1 drop of caprylic alcohol, and 2.5 ml. *excess* of freshly prepared 0.04 N $Na_2S_4O_6$ (2 ml. of 0.1 N KIO_3, 1 ml. of 10 per cent HCl, and a little KI are mixed and the solution is titrated first with 0.1 N $Na_2S_2O_3$ and finally with 0.02 N $Na_2S_2O_3$) to the digest after destroying the excess KH_2PO_2. Then a ground glass joint with a stop-cock attached is fitted to the digestion flask. The inflow of N_2, which has continued throughout all previous manipulations, is stopped and the flask is deaerated to 20 mm. of Hg. Then NH_4OH is added from a burette attached to the stopcock with a short length of rubber tubing, until the indicator has turned blue. 0.5 ml. excess of ammonia are added. The stopcock is

closed off and the vessel is allowed to stand at 40° for 15 minutes. Then 10 ml. of 5 N HCl are added and with the stream of N_2 resumed, the homocysteine is titrated according to Baernstein. c. The following corrections are suggested for methionine by the volatile iodide method, 1.067; by titration of homocysteine, 1.12; for cysteine, 1.023.

Kuhn, Birkofer, and Quackenbush (395) have modified the Baernstein method as follows: An extra washer is inserted ahead of those used by Baernstein to retain any I_2, this consists of 1 ml. of a 1 per cent suspension of red P to 9 ml. of water. At the end of the digestion, washer No. 3, saturated $HgCl_2$, is treated with an excess of $N/250$ I_2 and 0.5 ml. of 2 N HCl and after 30 minutes, the excess I_2 is titrated with $N/250$ $Na_2S_2O_3$ to determine the H_2S liberated during the hydrolysis.

Kuhn et al. (395) advise distilling the HI over KH_2PO_2 and under nitrogen. The reagent is prepared fresh each week and preserved with 1 per cent KH_2PO_2. They found that methionine yielded 94 per cent of the expected quantity of CH_3I.

Lavine (406) has shown that cysteine is quantitatively oxidized to cystine in M HI at room temperature. These conditions should, therefore, be approximated in the titration of cystine and of homocysteine.

B. Beach's Gravimetric Procedure (58)

Principle: The protein is hydrolyzed with HI. The hydrolysate contains, among other amino acids, cysteine and homocysteine thiolactone. The former is precipitated by cuprous oxide (Hopkins), the latter is not.

Reagents: HI is redistilled from the KH_2PO_2 preservative before use.

AgCl is prepared by adding dilute HCl to silver lactate solution. It must be kept wet and in a dark place.

Citrate buffer is made from 12 gm. of sodium citrate and 15 gm. of citric acid in 200 ml. of water. Then 20 ml. of glacial acetic acid are added and the solution is diluted with 9 volumes of water before use.

Procedure: 1. Hydrolysis. 0.5 to 1.0 gm. of protein (6 mg. of methionine) are hydrolyzed under reflux for 18 hours with 25 ml. of concentrated HI (under N_2). The excess HI is removed by concentration *in vacuo.* The addition of a few ml. of dilute HCl facilitates the removal of the excess HI.

2. Removal of HI. The hydrolysate is then transferred to a 250 ml. centrifuge bottle, volume 50 ml. An excess of AgCl suspension is added and the suspension is shaken in order to ensure the com-

plete removal of HI. The precipitate is centrifuged and washed twice with water and the filtrate is evaporated to a thick syrup. The residue is diluted to 50 ml.

3. Determination of Cysteine. To 25 ml. of the hydrolysate, 1 ml. of 20 per cent HCl and 300 mg. of Zn dust are added. After 2 hours, the solution is filtered and the p_H is adjusted to 4 to 5 by the addition of saturated CH_3COONa. Then, with mechanical stirring a slight excess of Cu_2O suspension is added. The solution is stirred 30 minutes longer, centrifuged, and the precipitate is washed 3 times with 30 ml. portions of the citrate-acetate buffer. Cystine is calculated by a sulfur determination on the mercaptide precipitate.

$$1 \text{ mg. of } BaSO_4 \backsimeq 0.515 \text{ mg. of Cystine.}$$

4. Determination of Methionine. The remainder of the hydrolysate is neutralized with 5 N NaOH and then 1 ml. excess of alkali is added. After 15 minutes, 2 ml. of 20 per cent HCl and 300 mg. of Zn dust are introduced. After standing over night, the solution is heated on the steam bath for 2 hours and the excess Zn is removed by decantation. A slight excess of Cu_2O suspension is added to the warmed (60°) solution, and after stirring for 15 seconds, the mercaptides are separated by centrifugation and washed with citrate-acetate buffer.

As the above procedure precipitates both cysteine and homocysteine, methionine is calculated from the non-cystine sulfur.

$$1. \text{ mg. of } BaSO_4 \backsimeq 0.709 \text{ mg. of Methionine (corrected).}$$

Comment: Beach and Teague (58) report recoveries of cystine to be from 80 to 100 per cent and of methionine from 87 to 92 per cent. They suggest that the methionine values be corrected by the factor 1.11.

It appears to the authors that certain modifications based on the findings of Rossouw and Wilken-Jorden and of Zittle and O'Dell would simplify and shorten this procedure. If cystine were determined by some other method; then nitrogen estimations should be satisfactory for the calculation of methionine, because any non-mercaptide nitrogen should be present in both precipitations in equal quantity and thus cancel the error.

C. The Polarographic Method of Stern and Beach (591)

Principle: Homocystine or homocysteine may be determined in a solution of 0.1 N NH_4OH, 0.1 N NH_4Cl, and 10^{-2} M $CoCl_2$ by the Heyrovsky polarograph provided that cystine, cysteine, and similar sulfur compounds are absent. Otherwise the polarograph gives the

sum of cystine and homocysteine (*cf.* Section 8, Part II of this Chapter).

<div align="center">*3. A Direct Colorimetric Method for Methionine
(McCarthy and Sullivan, 444)</div>

Principle: Methionine forms a colored compound with sodium nitroprusside.

Reagents: 14.3 N NaOH: Dissolve 57.2 gm. of NaOH in water. Dilute to 100 ml.

HCl–H₃PO₄ Mixture: Mix 9 volumes of concentrated HCl with 1 volume of 85 per cent H₃PO₄.

Method: 1. Hydrolysis. 500 mg. of protein are hydrolyzed with 2 ml. of 20 per cent HCl in an oil bath at 125° for 2 to 24 hours. The hydrolysate, after dilution, may be decolorized with 50 mg. of activated carbon (Carbox E). The carbon is washed with 5 ml. of hot and 5 ml. of cold N HCl. The filtrate and washings are adjusted to *p*H 3.5 with 5 N NaOH and the solution is diluted to 50 ml. with 0.1 N HCl.

2. Determination. To 5 ml. of unknown, the following reagents are added in order and with mixing after each addition, 1 ml. of 14.3 N NaOH, 1 ml. of 1 per cent glycine, and 0.3 ml. of 10 per cent sodium nitroprusside (freshly prepared). The tube is placed in a water bath at 35 to 40° for 5 to 10 minutes. Then it is cooled in ice water for 2 minutes and 5 ml. of HCl–H₃PO₄ mixture are added with mixing. It is shaken for one minute longer and cooled in water at room temperature for 5 to 10 minutes. The color is read against a closely matching standard or a calibration curve. Light filter 540 mu.

Comment: This reaction does not give any color with cystine, cysteine, homocystine or any other amino acid commonly present in protein hydrolysates except tryptophane. The latter is, however, destroyed during the acid hydrolysis. Although the color developed does not follow Beer's law, the reaction is quantitative over the range of 25 to 200 p.p.m. of methionine.

This simple and, if carefully conducted, accurate method has yielded excellent results in the authors' hands.

<div align="center">4. Iodometric Titration of Methionine (Lavine, 407, 408)</div>

Principle: An excess of iodine is added to a solution containing methionine. The remaining free I₂ is exactly removed with Na₂S₂O₃, the solution is acidified and the iodine bound to the methionine is liberated.

$$CH_3SCH_2CH_2CHNH_2COOH \xrightarrow[\underset{}{I_2}]{pH\ 7\ to\ 9} (CH_3S-R)^+I^- \xrightarrow[acid]{} CH_3SR+I_2$$

$$\underset{I}{\big|}$$
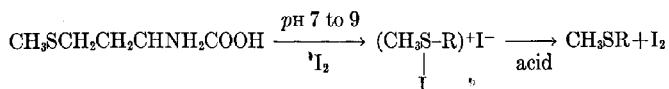
Reagents: Phosphate Buffer. 7 parts of M K_2HPO_4 and 3 parts of M KH_2PO_4.

Method: 1. Blank. 5 ml. of neutralized unknown (0.125 mM. of methionine), 1 ml. of 6 N HCl, and 3.8 ml. of 0.1 N KIO_3 are mixed and allowed to stand for 20 minutes. Then 1 ml. of 5 M KI and a mixture of 4 ml. of 5 M KI, 3 ml. of 2 N NaOH, 5 ml. of buffer, and 3 ml. of water are added. After standing, 20 minutes the I_2 is titrated with 0.025 N $Na_2S_2O_3$.

2. Determination. This is set up 10 minutes after beginning the blank. 5 ml. of neutralized unknown, 5 ml. of buffer, 5 ml. of 5 M KI, 7 ml. of water and 3.2 ml. of 0.1 N I_2 are mixed in order. After standing 20 minutes, the excess I_2 is removed with $Na_2S_2O_3$, 4 to 5 ml. of 2 N HCl are added and the iodine so liberated is titrated with 0.025 N $Na_2S_2O_3$.

1 mole $I_2 \eqsim$ 1 mole of Methionine.

Comments: It may be necessary to reduce the iodine concentration of the blank, before final titration with 0.025 N $Na_2S_2O_3$, to match that of the determination by adding a few drops of 0.2 M $NaHSO_3$.

Lavine (408) points out that the blank may be neglected in many cases. It is usually necessary to decolorize the protein hydrolysates with activated carbon (1:10).

5. TUTIYA'S METHYL SULFIDE PROCEDURE (623)

Principle: Methionine is fused with sodium hydroxide to yield CH_3SH. The latter is then aerated into a solution of isatin and the resulting green compound is determined colorimetrically.

Reagents: Isatin. 10 to 20 mg. of isatin are dissolved in 100 ml. of concentrated H_2SO_4.

Method: 0.2 to 100 mg. of methionine are fused for 1 to 2 minutes with 450 to 750 mg. of NaOH and a little water. The melt is then acidified with dilute HCl. The H_2S (from cystine) and CH_3SH (from methionine) are aerated first through a solution of lead acetate to remove the H_2S and then into the isatin-sulfuric acid reagent. A green color is formed.

Comment: Tutiya (623) claims that other amino acids and carbohydrates do not give any color under these conditions, but a mixture of cystine and betaine will do so.

PART IV

SULFUR AND SULFUR CONTAINING AMINO ACIDS IN PROTEINS

All values given in the following tables have been calculated to 16.0 per cent of nitrogen. In those instances, where nitrogen figures are not given by the authors, the amino acid values have been calculated using the value for N which is given in parenthesis. If the investigators reported their data in amino acid N as per cent of total N, then the results have been recalculated to 16.0 per cent of nitrogen, but the value of N is omitted from the tables.

Cf. Chapter I, Part VII for comments on "Best Values" and the calculation of the mean with twice the standard error.

Under the heading "Method," the general principle used to estimate or calculate cystine and methionine is given. The procedures used for sulfur are standard and are not given by name. Sulfur values based upon the Benedict-Denis procedure are known to be low (Painter, 509) and have been omitted from the tables.

The method adopted previously of referring to a procedure by the name of its original proponent has been knowingly modified in one instance, the reducing action of cysteine on phosphotungstic acid to give a blue color which was first reported by Winterstein, is, in conformity with usual practice, designated the Folin method.

ALBUMINOIDS

Sulfur Amino Acids in *Gelatins*, *Elastins*, and *Related Proteins*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Gelatin	Gasometric	Baernstein	39	(16.0)		0.0	
Gelatin	Folin	Baernstein	39	(16.0)		0.2	
Gelatin	Mörner-Okuda	Baernstein	39	(16.0)		0.1	
Gelatin	Gasometric, Baernstein	Baernstein	41	(16.0)	0.47	0.2	1.0
Gelatin	Hopkins-Beach	Beach	58	16.7	0.36	0.0	0.7
Gelatin	Folin	Folin	231	(16.0)		0.2	
Gelatin	Folin	Jones	342	(16.0)		0.2	
Elastin	Folin, Baernstein	Stein	586	17.1	0.16	0.2	0.4
Neurogelatin	Folin	unpublished		14.7	1.6	0.0	
Fish Gelatin	Fleming, McCarthy	unpublished		11.8		+	1.5

ALBUMINOIDS

The small quantities of the sulfur containing amino acids in the proteins of connective tissue are well known. Gelatin, although low

in methionine, does not appear to be devoid of this essential amino acid as is so often reported. One should remember however that the composition of "gelatin" will vary with its source and mode of preparation.

ANIMAL PROTEINS

Sulfur Amino Acids in *Entire Animals*

						Calculated to 16.0 gm. N.	
PROTEIN	METHOD	REFERENCE	NITRO-GEN	SUL-FUR	CYS-TINE	METH-IONINE	
			per cent	gm.	gm.	gm.	
Rat-Adult	Folin	unpublished	11.7		1.3		6 preps.
Rat-1 day old	Fleming, Calculated	unpublished	13.3	0.9	1.3	2-3	
Rat-23 day old	Fleming, Calculated	unpublished	11.1	1.1	1.7	3	
Rat-100 day old	Fleming, Calculated	unpublished	13.4	1.2	1.4	3	
Rat-18 mo. old	Fleming, Calculated	unpublished	12.2	1.3	2.0	3	
Rat-Adult	Folin	unpublished	13.1	1.3	1.2		a.
Rat-Adult	Folin	Roche 548	16.3	0.95	3.5		a.
Rat-Adult	Folin	Roche 548	16.7	0.93	3.4		b.
Rat-Adult	Folin	Roche 548	15.8	0.91	3.3		
Chick-Embryo	Folin	Calvery 141	(15.0)		2.8		

a Protein inanition
b Total inanition

ANIMAL PROTEINS

The discrepancies between our cystine values and those of Roche may be ascribed to differences in method; the Folin procedure will give high results in certain circumstances, or to an actual difference in the amino acid composition of the animals studied. In the case of cystine one thinks immediately of hair. This second explanation appears, however, to be ruled out by the determinations of total sulfur. It is the authors' opinion that the entire rat carcass contains approximately 2 per cent or less of cystine, for if Roche's values were correct almost the entire sulfur would be accounted for by cystine S.

BLOOD PROTEINS

Sulfur Amino Acids in *Fibrin*

					Calculated to 16.0 gm. N.	
SOURCE	METHOD	REFERENCE	NITRO-GEN	SULFUR	CYSTINE	METH-IONINE
			per cent	gm.	gm.	gm.
	Gasometric	Baernstein 39	(17.7)		1.3	
	Folin	Baernstein 39	(17.7)		3.2	
	Mörner-Okuda	Baernstein 39	(17.7)		1.3	
	Gasometric, Baernstein	Baernstein 41	(17.7)	0.88	1.5	2.2*
	Folin	Jones 342	(17.7)		3.4	
	Hopkins-Vickery	Vickery 652	(17.7)		1.4	
Beef	Schulz	Zahnd 695	(17.7)		1.5*	
Beef	Folin, McCarthy	unpublished			1.9	2.6
Average					1.9	

* Best Values

BLOOD PROTEINS

Sulfur Amino Acids in *Hemoglobins*

Calculated to 16.0 gm. N.

HEMO-GLOBIN	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Horse	Baernstein	Kuhn	395	(16.7)			1.0
Horse	Schulz	Schulz	573	(16.7)	0.41*	0.7*	
Horse	Hopkins-Vickery	Vickery	652	(16.7)		0.4	
Horse	Hopkins-Vickery	Vickery	653	16.7	0.37	0.4	
Horse	Schulz	Zahnd	695	(16.7)		0.7	
Cattle	Hopkins	Bergmann	67	17.0		0.5	
Cattle	Calculation	Block	94	16.1			1.4
Sheep	Calculation	Block	94	16.8			2.8
Sheep	Hopkins-Vickery	Vickery	653	16.8	0.70	0.6	
Dog	Baernstein	Kuhn	395	(16.4)			0.5
Dog	Hopkins-Vickery	Vickery	653	16.5	0.55	1.1	
Turtle	Folin, Calculation	unpublished		15.5	0.9	0.6	3
Pig	Folin, Calculation	unpublished		15.0	0.5	0.4	2

* "Best Values"

BLOOD PROTEINS

Sulfur Amino Acids in *Globins*

Calculated to 16.0 gm. N.

GLOBIN	METHOD	REFERENCE		NITRO-GEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Horse	Hopkins, Baernstein	Beach	56	16.4	0.39	0.8	0.7
Horse	Baernstein	Birkhofer	75	(16.4)		0.8	0.9
Horse	Baernstein	Kuhn	395	(16.4)			1.0
Horse	Folin	Roche	550	16.8	0.49	0.7	
Horse	Brdicka	Stern	590	16.4		0.8	
Horse	Schulz	Schulz	573	16.4	0.41	0.8	
Cattle	Hopkins, Baernstein	Beach	56	15.7	0.44	0.4	1.3
Cattle	Baernstein	Birkhofer	75	(15.7)		0.6	1.8
Cattle	Folin	Roche	550	16.6	0.50	0.6	
Cattle	Brdicka	Stern	590	15.7		0.4	
Sheep	Hopkins, Baernstein	Beach	56	16.1	0.56	0.8	1.2
Sheep	Folin	Roche	550	16.8	0.53	0.8	
Sheep	Brdicka	Stern	590	16.1		0.7	
Human	Hopkins, Baernstein	Beach	56	16.6	0.58	1.2	1.2
Human	Hopkins, Beach	Beach	58	16.7	0.65	1.2	1.5
Human	Baernstein	Birkhofer	75	(16.6)		1.3	1.5
Human	Fleming-Vassel	unpublished		16.2	0.6	0.8	
Human	Folin	Roche	550	16.7	0.68	0.8	
Human	Brdicka	Stern	590	16.6		1.1	
Ape	Baernstein	Birkhofer	75	(16.6)		1.2	1.3
Dog	Baernstein	Birkhofer	75	(16.0)		1.8	0.6
Dog	Baernstein	Kuhn	395	(16.8)			0.5
Dog	Folin	Roche	550	16.6	0.51	0.8	
Fox	Baernstein	Birkhofer	75	(16.0)		1.7	0.6
Jackal	Baernstein	Birkhofer	75	(16.0)		1.6	0.6
Pig	Hopkins, Baernstein	Beach	56	16.5	0.36	0.8	0.7
Pig	Folin	Roche	550	16.3	0.54	0.7	
Guinea Pig	Folin	Roche	550	16.7	0.60	0.8	

BLOOD PROTEINS

Sulfur Amino Acids in *Serum Albumins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Horse ?	Gasometric	Baernstein	39	(16.0)		3.9	
Horse ?	Folin	Baernstein	39	(16.0)		6.0	
Horse ?	Mörner-Okuda	Baernstein	39	(16.0)		1.6	
Horse	Folin, Baernstein	Brand	126	15.9	1.69	6.3	0.0
Horse ?	Brdicka	Brdicka	131	(16.0)		3.4	
Horse ?	Folin	Folin	234	(16.0)		6.1	
Horse-Fraction A	Folin	Hewitt	297			6.5*	
Horse-Fraction II	Folin	Hewitt	297			2.0*	
Horse		Mörner	463	(16.0)	1.29		
Horse	Folin	Reiner	543			5.9	
Horse-Crystalline	Schulz	Schulz	573	(16.0)	1.89	4.8	
Horse ?	Sullivan	Sullivan	596	(16.0)		5.7	
Horse ?	Hopkins	Zittle	698	(16.0)		5.9	
Horse ?	Hopkins-Sullivan	Zittle	698	(16.0)		5.7	
Cattle	Folin	Reiner	543			4.0	
Human	Folin	Alving	30	14.6		5.7	
Human-Na₂SO₄	Folin	Bálint	46	16.0		5.0±.3*	
Human-Na₂SO₄	Sullivan	Murrill	469	13.5	•	4.8	
Human	Folin	Reiner	543			6.0	
Human	Folin	Tuchman	621	(16.0)		6.1±1.1	

* "Best Values"

BLOOD PROTEINS

Sulfur Amino Acids in *Serum Globulins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Horse ?	Brdicka	Brdicka	131	(16.0)		1.6	
Horse[a]	Folin, Calculation	Calvery	144	15.9	1.3	3.1	2
Horse[b]	Folin, Calculation	Calvery	145	(16.0)	1.3	2.6	3
Horse ?		Mörner	463	(16.0)	0.67		
Horse	Folin	Reiner	543			3.4	
Horse	Schulz	Schulz	573	(16.0)		2.4	
Horse	Sullivan	Sullivan	598	(16.0)		1.9	
Cattle	Folin	Reiner	543			2.9	
Human	Folin	Alving	30	14.6		2.4	
Human	Sullivan	Alving	30	14.6		2.0	
Human	Mörner-Okuda	Alving	30	14.6		2.1	
Human-Na₂SO₄	Folin	Bálint	46	16.0		2.5±.3*	
Human-Na₂SO₄	Sullivan	Murrill	469	14.0		3.4	
Human	Folin	Reiner	543			3.5	
Human	Folin	Tuchman	621	(16.0)		3.6±.8	

* "Best Values"
a Pneumococcus precipitate type II
b Pneumococcus precipitate type I

BLOOD PROTEINS

Sulfur Amino Acids in *Total Serum Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Human	Folin	Bálint	46	16.0		3.5 ± .4	
Human	Folin	Block	105	14.9	1.57	3.9	
Human	Folin, Calculation	Block	110	15.4	1.34	3.4	2.0
Human	Folin, Calculation	Block	110	15.2	1.34	3.4	2.0
Human	Sullivan, Calculation	Murrill	469	14.3	1.62	4.3	2.2
Human	Folin	Tuchman	621	(16.0)		4.8 ± .6	
Human-Myeloma	Folin, Calculation	unpublished		14.1	1.2	2.9	2
Human-Phenylpyruvic	Folin	Block	105	14.9	1.55	3.2	
Human-Nephritic	Sullivan, Calculation	Murrill	469	14.7	1.62	4.2	2.3
Dog	Sullivan	Murrill	470	12.2	1.17	3.3	
Dog-Reserve Protein	Sullivan	Murrill	470	11.6	1.15	3.2	
Dog-Regenerated	Sullivan	Murrill	470	14.4	1.20	3.1	
Dog-Casein diet	Sullivan	Murrill	470	12.7	1.11	3.1	
Dog-Lactalbumin diet	Sullivan	Murrill	470	12.2	1.14	3.1	
Dog-Beef serum diet	Sullivan	Murrill	470	14.1	1.34	3.3	
Dog-Yeast diet	Sullivan	Murrill	470	14.2	1.19	3.1	
Cattle ?	Hopkins-Graff	Graff	264			3.6	
Mean with 2×S.E.					1.32 ± 0.10	3.6 ± .1	2.1

BLOOD PROTEINS

Sulfur Amino Acids in *Urine Proteins*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Bence-Jones	Folin, Calculation	Calvery	143	18.0	0.89	2.7	0.8
Bence-Jones	Baernstein	Devine	195	14.7	1.46	3.2	0.6
Bence-Jones	Folin	Folin	231	(16.0)		3.5	
Bence-Jones		Hopkins	308	16.2	1.17		
Nephritic	Sullivan	Murrill	469	15.1	1.86	5.9	

BLOOD PROTEINS

Sulfur Amino Acids in *Stroma Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Sheep	Hopkins, Baernstein	Beach	55	14.0	0.86	1.1	2.4
Horse	Hopkins, Baernstein	Beach	55	12.9	0.89	1.1	2.4
Hog	Hopkins, Baernstein	Beach	55	13.1	0.87	1.4	2.1
Beef	Hopkins, Baernstein	Beach	55	13.8	0.82	0.9	1.7
Beef	Hopkins	Erickson	213	13.8		1.0	
Beef-Embryo	Hopkins	Erickson	213	13.7		1.4	
Human	Hopkins, Baernstein	Beach	55	13.0	0.88	1.1	2.2
Human[a]	Hopkins	Erickson	213	13.1		1.4	
Human[b]	Hopkins	Erickson	213	13.7		1.3	

[a] Polycythemia vera
[b] Polycythemia cardiac

BLOOD PROTEINS

Hemoglobins and Globins: There are considerable differences in the absolute quantities and relative proportions of sulfur, cystine, and methionine in globins and hemoglobins of different species. In fact these relatively large differences in the sulfur amino acids of globins are in marked contrast to the relatively small specie differences in arginine, histidine, lysine, tyrosine, tryptophane, and phenylalanine.

Serum Albumins: As mentioned before, the composition of any serum albumin fraction, even though homogeneous and crystalline, is a function of the method used in its preparation. The use of data from the literature to compute the molecular weight, acid and base binding capacity, etc. of any serum protein fraction must be made circumspectly unless the fraction analyzed and that under physico-chemical study, has been shown to be identical by phase rule studies, etc. There have been numerous such calculations in the recent literature in which this has not been done and consequently are misleading to the average reader.

Serum Globulins: These are even more inhomogeneous proteins than are the serum albumins. However, it is apparent that serum globulins contain only about one half to one third the quantity of cystine present in serum albumins.

Serum Proteins: Amino acid analyses of total serum proteins in health and disease is a more valuable approach to protein changes under these conditions. The serum proteins from a patient with multiple myeloma contained less cystine than normal. This is to be expected in view of the practically complete disappearance of serum albumin in this case.

Urine Proteins: The decrease in serum albumin is even more marked in multiple myeloma than in nephritis, yet Bence-Jones protein in contrast to the urinary protein in nephritis is relatively low in cystine.

Stroma Proteins: There does not appear to be any distinct species specificity among these proteins with respect to sulfur and cystine. This is in contrast with the findings on globins.

BRAIN PROTEINS

Sulfur Amino Acids in *Brain Proteins* (*cf.* 59, 99, 354, 105, and unpublished results)

Calculated to 16.0 gm. N.

ANIMAL	METHOD	NITROGEN	SULFUR	CYSTINE	METH-IONINE	
		per cent	gm.	gm.	gm.	
Human-Males	Folin, Calculation	13.9	1.2	2.1	3	17 cases
Human-Females	Folin, Calculation	13.8	1.2	2.1	3	6 cases
Monkey-Males	Folin	14.2		2.0		6 cases
Monkey-Females	Folin	14.5		2.1		8 cases
Sheep-Males	Folin	13.7		2.0		7 cases
Sheep-Females	Folin	14.3		1.9		4 cases
Rat	Folin	14.5		2.1		5 cases
Guinea Pig	Folin	13.8		1.6		2 cases
Cattle	Folin	14.2		2.2		2 cases
Cattle	Hopkins, McCarthy		1.03	2.0	3.0	
Pig	Folin	15.4		1.9		2 cases
Rabbit	Folin	12.8		2.2		2 cases
Cat	Folin, Mörner	15.2		1.4		(354)
Dog	Folin, Mörner	15.1		1.4		(354)
Frog	Folin, Mörner	15.3		1.2		(354)
Sheep	Folin, Mörner	15.2		1.4		(354)
Mean with 2×S.E.				1.8± .2		

EGG PROTEINS

Sulfur Amino Acids in *Crystalline Egg Albumin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
			per cent	gm.	gm.	gm.
Gasometric	Baernstein	39	(15.4)		1.6	
Folin	Baernstein	39	(15.4)		1.3	
Gasometric, Baernstein	Baernstein	41	(15.4)	1.66	2.4	4.8
Baernstein-Iodide	Baernstein	42	(15.4)			5.3
Baernstein-Titration	Baernstein	42	(15.4)			4.7
Folin	unpublished		13.9	1.8	2.4	
Brdicka	Brdicka	131	(15.4)		2.1	
Folin	Calvery	139	(15.4)		1.4	
Folin	Folin	234	(15.4)		1.3	
Heffter-Guthrie	Guthrie	275	(15.4)		1.3[a]	
Hellerman	Hellerman	288	(15.4)		1.4	
Sullivan	Hess	291	(15.4)		1.3	
Folin	Jones	342	(15.4)		0.9	
Baernstein	Kassell	357	14.9	1.94	1.9	5.6
Baernstein	Kuhn	395	(15.4)		1.9	5.0*
Lavine	Lavine	408	(15.4)			4.8
Schulz	Schulz	573	(15.4)	1.18	1.8*	
Sullivan	Sullivan	596	(15.4)		1.3	
Folin	Tompsett	615	(15.4)		2.3	
Baernstein	Virtanen	663	12.1			4.8
Schulz	Zahnd	695	(15.4)		1.7	
Mean with 2×S.E.				1.65	1.7± .2	5.0± .3

* "Best Values"

[a] 0.6 per cent cysteine

EGG PROTEINS

Sulfur Amino Acids in *Egg Proteins* Other Than Albumin

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Egg White	Gasometric	Baernstein	39	(15.0)		2.2	
Egg White	Mörner-Okuda	Baernstein	39	(15.0)		2.1	
Egg White	Hopkins, Beach	Beach	58	15.0	1.9	3.2	4.4
Egg White	Folin	unpublished		14.8	1.8	2.6	
Egg White		Blumenthal	111	(15.0)	1.7		
Egg White	Folin	Calvery	141	(15.0)		1.8	
Egg White	Hopkins-Graff	Graff	264			2.8	
Egg White	Folin	McFarlane	448	15.2		2.3	
Egg White	Sullivan	Prunty	530	14.0		1.7	
Mean with 2×S.E.					1.8	2.3±.4	4.4
Conalbumin	Folin	Jones	342	(15.4)		3.5	
Egg Yolk	Folin, Calculation	unpublished		14.6	1.2	1.9	3
Egg Yolk	Folin, Calculation	unpublished		15.0	1.0	1.9	2-3
Egg Yolk	Folin	Calvery	141	(15.0)		1.4	
Egg Yolk	Folin	McFarlane	448	15.2		2.3	
Whole Egg	Folin, Calculation	unpublished		14.1	1.5	2.4	4
Vitellin	Baernstein-Iodide	Baernstein	42	(15.0)			2.9
Vitellin	Baernstein-Titration	Baernstein	42	(15.0)			2.8
Vitellin	Folin	Calvery	140	15.0	1.0*	1.3	
Vitellin	Folin	Jones	342	16.3		0.8	
Vitellin	Sullivan	Jukes	347			1.2	
Vitellin	Baernstein	Kuhn	395	(16.0)		1.8	3.1
Livetin	Baernstein-Iodide	Baernstein	42	(15.5)			2.4
Livetin	Baernstein-Titration	Baernstein	42	(15.5)			2.4
Livetin	Sullivan	Jukes	347	14.8		2.5	
Livetin	Folin	Jukes	348	15.5		3.6	
Ovomucoid	Baernstein-Iodide	Baernstein	42	(13.5)			1.7
Ovomucoid	Baernstein-Titration	Baernstein	42	(13.5)			1.6
Ovomucoid	Folin	McFarlane	448	13.5	2.7	6.2	

EGG PROTEINS

Schulz (573) in 1898 was the first to point out the relatively small proportion of total sulfur which was split off from egg albumin by alkaline lead acetate. He apologized for this low value by saying that he had sufficient crystalline egg albumin for one determination only. It is therefore especially interesting that his value for labile sulfur, when calculated as cystine, agrees with the best values in the literature at present.

The high sulfur content of egg proteins, especially egg white proteins, has long been recognized.

FOODS

Sulfur Amino Acids in *Feeds* and *Foods*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITRO-GEN	SULFUR	CYSTINE	METH-IONINE	
			per cent	gm.	gm.	gm.	
Bread	Folin, Calculation	unpublished	11.2	1.1	2.2	2–3	6% milk solids
Bread	Folin, Calculation	unpublished	11.3	1.2	2.0	3	6% milk solids
Flour	Folin, Calculation	unpublished	12.8	1.1	1.9	3	5 samples
Cereal	Fleming, Folin	unpublished		1.0	1.2		"Wheatena"
Cereal	Fleming, Folin	unpublished	12.3	1.4	1.8		"Ralston"
Cereal	Fleming, Folin	unpublished		1.3	1.5		"Cream Farina"
Cereal	Fleming, Folin	unpublished	13.6	1.1	1.7		"Cream of Wheat"
Cereal	Fleming, Folin	unpublished	13.8	1.1	1.4		"New Cream of Wheat"
Cereal	Fleming, Folin	unpublished		1.1	0.7		"Puffed Sparkies"
Cereal	Folin, McCarthy	unpublished			0.7	1.6	"Cerevim"
Linseed Meal	Folin, Calculation	unpublished		1.1	1.9	3	
Alfalfa Leaf Meal	Folin, Fleming	unpublished	10.6	1.3	1.4		
Soybean Meal	Folin	unpublished			1.3		
Soybean Meal	McCarthy-Sullivan	Almquist 29				2.0	
Flaxseed Meal	Folin, McCarthy	unpublished			1.9	2.3	
Peanut Meal	Folin, McCarthy	unpublished	10.4		1.6	0.9	
Cottonseed Meal	Folin, McCarthy	unpublished	10.9		2.0	1.6	Proflo
Meat Scraps	Folin, Calculation	unpublished		1.1	1.0	3	
Tankage	Folin, Calculation	unpublished	10.6	0.8	0.9	3	
Menhaden Meal	Folin, Calculation	unpublished	11.6	0.9	1.0	3	
Haddock Meal	Folin	Pottinger 526			1.1		

FEEDS AND FOODS

Cereals: The apparent injurious action of "puffing" on the cystine content of a wheat cereal should be noticed.

Peanut Meal: As was to be expected from the analyses of arachin, peanut meal proteins are the lowest in methionine content of any proteins so far reported with the exception of gelatin and wool.

HORMONES AND ENZYMES

Sulfur Amino Acids in *Hormones* and *Nonmetalic Enzymes*

PROTEIN	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METHIONINE
				per cent	gm.	gm.	gm.
Insulin	Baernstein	Kuhn	395	15.7		12.2	0.6
Insulin	Folin, Baernstein	Miller	455	15.5	3.45	12.9± .4*	0.0*
Insulin	Brdicka	Sullivan	600	(15.7)		12.9—13.6	
Insulin	Sullivan	Sullivan	600	(15.7)		11.8—12.8	
Insulin	Fleming-Vassel	Vassel	641	(15.7)		10.8	
Insulin	Fleming-Vassel	Vassel	641	(15.7)		10.3	
Insulin	Folin	du Vigneaud	660	15.7		13.1	
Insulin	Sullivan	du Vigneaud	660	15.7		8.4	
Insulin	Baernstein	du Vigneaud	660	15.7		12.4	0.0
Pepsin	Folin	Calvery	146	15.4		1.5	
Pepsin	Folin	Calvery	146	15.2		1.3	Heat coagulum
Pepsin	Folin	Calvery	146	15.4		2.3	Heat filtrate
Thyroglobulin	Baernstein	Brand	122	(15.8)	1.47	4.4	1.3
Thyroglobulin	Folin	Eckstein	202			1.6	
Chymotrypsinogen	Sullivan, Baernstein	Brand	125	16.2	2.1	4.5	1.2
Pituitary Lactogenic		Li	419	(16.0)		3.0	
Pituitary Oxytocic	Sullivan	Potts	528	(16.0)	5.59	18.3	
Pituitary Pressor	Sullivan	Potts	528	(16.0)		19.0	
Rous Sarcoma	Folin, Fleming	unpublished		14.6		5.2	
Secretin	Sullivan	Ågren	25	14.4	0.8	0.0	

Calculated to 16.0 gm. N.

* "Best Values."

ANIMAL HORMONES AND ENZYMES

Insulin: It is interesting that insulin is relatively rich in cystine and devoid of methionine just as it is abundantly supplied with tyrosine but devoid of tryptophane.

Pressor and Oxytocic Hormones: The large amounts of cystine present in these biologically active substances should be noticed.

KERATINS

Sulfur Amino Acids in *Human Hair*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	
			per cent	gm.	gm.	
Folin	Block	97	15.4	5.2	16.1	
Folin	Block	109	16.6	4.7	15.0	
Brdicka	Brdicka	131	(16.6)		17.0	brown
Brdicka	Brdicka	131	(16.6)		17.2	blond
Brdicka	Brdicka	131	(16.6)		16.4	child
Brdicka	Brdicka	131	(16.6)		13.6	red
Folin	Clay	166	15.5	5.47	17.4	male
Sullivan	Clay	166	15.5		15.3	male
Folin	Clay	166	15.4	5.47	16.2	female
Sullivan	Clay	166	15.4		14.1	female
Sullivan	Clay	166				0.5% cysteine
Folin	Folin	231	(16.6)		15.9	
Sullivan	Lewis	418	17.3		14.8±1.2	
Isolation	Mörner	463	(16.6)	3.92	13.4	
Folin	Wilson	681	15.4		19.6	children
Folin	Wilson	681	15.4		18.3	adults
Mean with 2×S.E.					15.9±.9	

KERATINS

Sulfur Amino Acids in *Sheep's Wool*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
			per cent	gm.	gm.	gm.
Isolation	Abderhalden	16	(16.6)		7.0	
Gasometric	Baernstein	39	(16.6)		9.9	
Folin	Baernstein	39	(16.6)		7.5	
Mörner-Okuda	Baernstein	39	(16.6)		8.8	
Sullivan, Baernstein	Bailey	43	16.3	3.56	12.3	0.6
Folin	Bailey	43	16.3		12.3	
Sullivan, Baernstein	Bailey	43	16.2	3.69	11.5	0.6
Folin	Bailey	43	16.2		11.6	
Baernstein	Barritt	52	16.6	2.9–3.8		0.5±.1
Folin	Block	77	16.6	4.38	9.6	
Folin, Calculated	Block	97	15.4	3.7	13.6	0.5
	Blumenthal	111	(16.6)	3.0		
Brdicka	Brdicka	131	(16.6)		9.3	
Folin	Folin	231	(16.6)		7.5	
Sullivan	Gordon	261			10.8	
Hopkins-Graff	Graff	264			9.6	
Sullivan	Hess	293	(16.6)		13.1	
Mörner-Okuda	Hess	293	(16.6)		13.4	
Folin-Shinohara	Hess	293	(16.6)		13.6	
Folin-Marenzi	Hess	293	(16.6)		15.3	
Hopkins-Sullivan	Hess	293	(16.6)		13.3	
Isolation	Martin	437				0.7
Sullivan	Sullivan	596	(16.6)		12.4	
Sullivan	Sullivan	601	(16.6)	3.2	11.3	
Mörner-Okuda	Sullivan	601	(16.6)		11.5	
Folin	Sullivan	601	(16.6)		12.5	
Hopkins-Vickery	Vickery	652	(16.6)		9.2	
Baernstein	du Vigneaud	661	(16.6)			0.5
Folin	Wilson	681			9.9	
Mean with 2×S.E.				3.5	11.1±.9	0.6

KERATINS

Sulfur Amino Acids in *Hair Proteins* other than Human Hair and Wool

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	SULFUR	CYSTINE	METH-IONINE	
				per cent	gm.	gm.	gm.	
Kemp	Sullivan, Baernstein	Bailey	43	15.9	3.37	11.2	0.8	
Kemp	Folin	Bailey	43	15.9		11.5		
Kemp	Sullivan, Baernstein	Bailey	43	15.5	1.96	1.9	0.8	scoured
Kemp	Folin	Bailey	43	15.5		2.0		scoured
Rat Hair	Folin	unpublished		14.7		13.4		a
Rat Hair	Folin	unpublished		14.8		15.6		b
Rat Hair	Folin	unpublished		14.6		16.0		c
Rat Hair	Folin	unpublished		14.7		14.7		d
Rat Hair	Folin	Wilson	681	(15.4)		14.6		
Camel Hair	Folin	Block	97	15.1	3.3	11.7		
Chimpanzee Hair	Folin, Calculated	Block	109	16.7	4.1	14.9	0.7	
Cattle Hair	Folin, Calculated	Block	109	15.5	3.8	13.9	0.6	
Cat Hair	Folin	Wilson	681	(15.4)		13.6		
Dog Hair	Folin	Wilson	681	(15.4)		19.7		
Hog Hair	Folin	unpublished		15.1	3.7	9.2		
Hog Hair	Fleming-Vassel	unpublished		15.1		8.3 ,		
Goat Hair	Folin	Block	97	16.2	3.1	8.8		
Rabbit Hair	Folin	Wilson	681	(15.4)		13.5		
Rabbit Hair	Lugg, McCarthy	unpublished				16.7	0.5	

a 30 per cent Milk Powder Diet
b 30 per cent Milk Powder Diet +cystine
c 30 per cent Milk Powder Diet +methionine
d 40 per cent Milk Powder Diet

KERATINS

Sulfur Amino Acids in *Horn Proteins* and similar Eukeratins

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE
				per cent	gm.	gm.
Cattle Horn	Isolation	Abderhalden	16	(15.1)		7.9
Cattle Horn	Folin	Block	97	16.1	2.8	8.2
Cattle Horn		Blumenthal	111	(16.1)	3.6	
Cattle Horn	Folin	Folin	231	(16.1)		6.7
Cattle Horn	Isolation	Mörner	463	(16.1)	2.48	6.8
Rhinoceros Horn	Folin	Block	97	15.6	2.4	8.9
Steer Hoofs	Folin	unpublished		15.0	2.0	6.3
Steer Hoofs	Fleming-Vassel	unpublished		15.0		6.0
Emu Bill	Folin	unpublished		13.2		4.7
Goose Bill	Folin	unpublished		10.6		6.3
Iquana Bill	Folin	unpublished		13.5		6.8
Porcupine Quills	Folin	Block	97	15.8	3.0	9.5
Echidna Quills	Folin	Block	97	15.2	4.0	12.5
Human Nails	Rossouw-Sullivan	Freyberg	243	(16.0)		11 ± 1
Human Nails	Sullivan	Lewis	418	16.8	.	10 ± 1
Human Nails	Sullivan	Hess	292	(14.9)		12.9
Goose Feathers		Block	77	15.5	3.3	
Goose Feathers	Folin	Wilson	681	15.1	3.0	10.5
Hen Feathers	Folin	Block	97	15.5	2.4	7.0
Turkey Feathers	Folin	Wilson	681	15.5	2.5	8.3
Duck Feathers	Folin	Wilson	681	15.7	3.0	10.7
Snake Skin	Folin	Block	97	15.2	2.3	6.9
Egg Shell Membrane	Folin	Calvery	141	(16.6)		6.2
Egg Shell Membrane	Folin	Calvery	142	16.6	3.64	12.2
Egg Shell Membrane	Isolation	Mörner	463	(16.0)	2.47	7.6

KERATINS

Sulfur Amino Acid in *Pseudokeratins*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITRO-GEN	SULFUR	CYSTINE	
				per cent	gm.	gm.	
Neurokeratin	Isolation	Argiris	32	14.2		1.7	
Neurokeratin		Block	78	14.1	2.25		
Neurokeratin	Folin	unpublished		13.3	2.4	4.1	
Neurokeratin	Folin	unpublished		10.9		4.5	Papain
Neurokeratin	Folin	unpublished		10.0		4.5	Pepsin
Neurokeratin	Folin	unpublished		8.3		3.9	Trypsin
Skin Human	Folin	Block	79	15.5	1.72	3.5	
Skin Human	Folin	Eckstein	203	14.2		4.3	
Skin Human	Folin	Wilkerson	678	15.1		2.3	
Skin Human	Folin, Baernstein	Wilkerson	679	15.1	1.15	2.5	2.6
Skin Human	Folin	Wilson	681	13.0	0.86	2.6	
Skin Lamb	Sullivan	Sullivan	601	(16.0)	0.79	0.9	
Skin Lamb	Mörner-Okuda	Sullivan	601	(16.0)		0.9	
Shell Tortoise	Folin	Wilson	681	(15.0)		7.9	
Scutes Turtle	Folin	Block	96	14.1	2.6	9.6	
Excrescence Pelican	Folin	Block	96	14.0	1.8	4.6	
Baleen Whale	Folin	Block	96	14.1	3.9	10.8	
Casing Fish Egg	Sullivan	Young	694	15.3		1.9	Salmon
Gorgonin	Folin, Hopkins	Block	96	14.1		10.2	G. flabellum
Gorgonin	Folin, Hopkins	Block	96	13.7		8.9	P. dichotoma
Spongin	Folin, Hopkins	Block	96	14.0		3.4	

KERATINS

Hair and Wool: One of the earliest recognized characteristics of keratins was their high content of sulfur and cystine. It is interesting that in spite of the large number of cystine determinations which have been carried out on eukeratins, there are only a few values for methionine, not a single one in the case of human hair. The noncystine S of hair may, however, indicate the presence of some methionine in this protein.

Variation in the cystine content of samples of the same protein can be due to many factors among the most significant is the treatment to which the protein was subjected before hydrolysis. Thus Bailey (43) has shown that "scouring" of kemp reduces its cystine to approximately one fifth of the initial value.

Pseudokeratins: The majority of the pseudokeratins yield less cystine than the average eukeratin.

LIVER PROTEINS

Sulfur Amino Acids in *Liver Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	SULFUR	CYSTINE	METH-IONINE	
				per cent	gm.	gm.	gm.	
Beef	Folin, Calculated	unpublished		13.3	1.1	1.4	3.4	
Beef	Hopkins, Beach	Beach	59		0.9	1.3	3.2	
Rat	Folin	Lee	411		1.0	1.8		
Cat	Folin	Urban	625	15.4	1.2	1.8		Albumin
Cat	Folin	Urban	625	14.8	0.82	1.2		Globulin
Cat	Folin	Urban	625	15.0	0.93	1.3	.	
Human	Folin	Block	105	13.6		1.6		
Normal	Baernstein	Greenstein	266	15.7	1.1	1.4±.1	3.2±.1	Nucleo-protein
Tumor #31	Baernstein	Greenstein	267	15.5	1.1	1.4	3.0	Nucleo-protein
Cod	Folin, McCarthy	unpublished				1.1	3.4	

METALLOPROTEINS

Sulfur Amino Acids in *Metalloproteins* other than Hemoglobin

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE	
				per cent	gm.	gm.	gm.	
Cytochrome	Folin	Theorell	605	15.4		0.7[a]		
Hemocyanin	Folin	Roche	550	15.6	1.0	2.0		Mollusc
Hemocyanin	Folin	Roche	550	16.8	1.0	1.8		Crustacea
Hemocyanin	Baernstein	Mazur	441	17.5	1.12	1.8	2.4	
Hemerythrin	Folin	Roche	550	16.8	1.66	2.4		Siphuncle
Ferritin	Baernstein	Kuhn	396	8.4		2.1	2.8	

[a] One mole of cysteine would require the presence of 1.9 per cent of cystine, *i.e.* a large hydrolytic loss is indicated or the preparation is inhomogeneous.

MILK PROTEINS

Sulfur Amino Acids in *Casein* from Cow's Milk

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
	Gasometric	Baernstein	39	(15.4)		1.0	
	Folin	Baernstein	39	(15.4)		0.3	
	Mörner-Okuda	Baernstein	39	(15.4)		0.3	
	Gasometric, Baernstein	Baernstein	41	(15.4)	0.88	0.7	3.5
	Baernstein-Iodide	Baernstein	41	(15.4)			3.4
	Baernstein-Titration	Baernstein	41	(15.4)			3.2
	Hopkins, Baernstein	Beach	55	15.1		⌈0.3	3.2
	Hopkins, Baernstein	Beach	57	14.5	0.88	⌊0.3	3.4
	Hopkins, Beach	Beach	58	15.8	0.85	0.3	3.2
Harris	Folin	unpublished		14.8	0.7		
Labco	Folin, Calculation	unpublished		15.8	0.7	0.5	2.7
Labco Hydrolyzed	Folin, McCarthy	unpublished		12.2		0.3	4.7
Labco Hydrolyzed	Folin, McCarthy	unpublished		12.3		0.3	4.9
Difco Hydrolyzed	McCarthy	unpublished		7.4		■	3.2
Difco Hydrolyzed	McCarthy	unpublished		10.5			3.7
		Blumenthal	111	(15.4)	0.65		
	Sullivan, Baernstein	Brand	121	14.8		0.4	3.5
	Sullivan	Csonka	181			0.3	

MILK PROTEINS (Continued)

Sulfur Amino Acids in *Casein* from Cow's Milk

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Hammersten	Folin	Folin	231	(15.4)		0.3	
Hammersten	Folin	Folin	234	(15.4)		0.2	
Cohn	Folin	Folin	234	(15.4)		0.3	
Hammersten	Hopkins-Graff	Graff	264			0.8	
Vitamin Free	Hopkins-Graff	Graff	264			0.2	
	Sullivan	Hess	291			0.3	
	Sullivan	Hess	293	(15.4)		0.3	
	Mörner-Okuda	Hess	293	(15.4)		0.3	
	Folin-Shinohara	Hess	293	(15.4)		0.5	
	Folin-Marenzi	Hess	293	(15.4)		0.5	
	Hopkins	Hess	293	(15.4)		0.3	
	Baernstein	Kuhn	395	(15.4)		0.5	3.2
	Lavine	Lavine	408	15.3			3.0
	Folin	Marenzi	436	(15.4)		0.3	
	Sullivan, McCarthy	McCarthy	444	15.1		0.5	3.3
	Baernstein	Plimmer	521	15.2		0.4	3.1
	Folin	Plimmer	521	15.2		0.3	
	Sullivan	Pottinger	527	(15.4)		0.3	
	Sullivan	Prunty	530	(15.4)		0.3	
Hammersten	Sullivan	Prunty	530	(15.4)		0.2	
Glaxo	Sullivan	Prunty	530	(15.4)		0.1	
	Brdicka	Stern	590	(15.4)		0.3	
	Hopkins	Stern	590	(15.4)		0.3	
	Sullivan	Sullivan	598	(15.4)		0.3	
	Baernstein	Toennies	613	14.1		0.4	3.0
	Folin	Tompsett	615	(15.4)		0.3	
	Fleming	Vassel	641	(15.4)		0.3	
Hammersten	Hopkins	Vickery	652	(15.4)		0.2	
Harris	Hopkins	Vickery	652	(15.4)		0.2	
Commercial	Hopkins	Vickery	652	(15.4)		0.5	
	Schulz	Zahnd	695	(15.4)		0.3	
Mean with 2×S.E.					0.78	0.36±.04	3.5±.3

MILK PROTEINS

Sulfur Amino Acids in *Lactalbumin*

			Calculated to 16.0 gm. N.		
METHOD	REFERENCE	NITROGEN	SULFUR	CYSTINE	METHIONINE
		per cent	gm.	gm.	gm.
Gasometric, Baernstein	Baernstein 41	(15.2)	1.64	4.0	2.8
Baernstein-Iodide	Baernstein 42	(15.2)			2.6
Baernstein-Titration	Baernstein 42	(15.2)			2.4
Hopkins, Baernstein	Beach 57	14.2	1.42	2.8	3.1
Hopkins, Beach	Beach 58	15.7	1.58	3.3	3.0
	Blumenthal 111	(15.2)	1.3		
Sullivan, Baernstein	Brand 121	14.8		3.4	3.0
Hopkins-Graff	Graff 264			3.0	
Sullivan	Hess 291	(15.2)		2.4	
Sullivan	Hess 293	(15.2)		2.3	
Mörner-Okuda	Hess 293	(15.2)		2.7	
Folin-Shinohara	Hess 293	(15.2)		2.8	
Folin-Marenzi	Hess 293	(15.2)		3.0	
Hopkins-Vickery	Hess 293	(15.2)		2.7	
Folin	Jones 342	(15.2)		4.1	
Baernstein	Kassell 357	14.6	1.55	3.4	3.1
Lavine	Lavine 408	(15.2)			2.6
Baernstein	Plimmer 521	14.2		3.7	2.6
Folin	Plimmer 521	14.2		2.9	
Sullivan	Sullivan 596	(15.2)		2.7	
Sullivan	Sullivan 598	(15.2)		2.3	
Fleming	Vassel 641	(15.2)		2.8	
Hopkins	Vickery 652	(15.2)		2.7	
Mean with 2 ×S.E.			1.50	3.0± .2	2.8± .2

MILK PROTEINS

Sulfur Amino Acids in *Milk Proteins* other than Casein and Lactalbumin

			Calculated to 16.0 gm. N.		
PROTEIN	METHOD	REFERENCE	NITROGEN	SULFUR	CYSTINE	METHIONINE
			per cent	gm.	gm.	gm.
Casein, Human	Hopkins, Baernstein	Beach 57	14.6	0.75	0.8*	2.4*
Casein, Human	Baernstein	Plimmer 521	14.4		0.7	3.0
Casein, Human	Folin	Plimmer 521			0.6	
Whole Milk, Human	Folin, McCarthy	unpublished	15.2	1.6	3.4	2.0
Whole Milk, Cow	Folin, McCarthy	unpublished	15.2	1.0	1.2	2.8
Whole Milk, Cow	Sullivan	Prunty 530	(4.0)		0.7	
Lactalbumin, Human	Hopkins, Baernstein	Beach 57	13.7	1.44	3.6± .4*	2.2*
Lactalbumin, Human	Baernstein	Plimmer 521	14.6		4.5	1.5
Lactalbumin, Human	Folin	Plimmer 521	14.6		3.2	
β-Lactoglobulin	Folin, McCarthy	Bolling 112	15.5	1.74	3.6*	3.9*
β-Lactoglobulin	Fleming-Vassel	Bolling 112	15.5		3.5	
β-Lactoglobulin	Folin, Baernstein	Brand 128	15.6	1.64	3.7	3.3

* "Best Values."

MILK PROTEINS

The differences in cystine and methionine contents of human and cow's milk are of special interest. In spite of the fact that cow's

lactalbumin contains somewhat more methionine than lactalbumin from human milk, the marked deficiency in cystine in casein from cow's milk makes the total proteins from the latter somewhat deficient with respect to the sulfur containing amino acids as compared to human milk proteins. This analytical observation has been checked by feeding experiments in white rats. If cystine or methionine is added to cow's milk in such proportions that the sulfur amino acid content is equivalent to human milk proteins there is a distinct increase in the nutritive value of the cow's milk proteins as shown by growth, nitrogen balance, and paired feeding experiments (cf. Keratin Table: Sulfur Amino Acids in *Hair Proteins* other than Human Hair and Wool, page 185).

MUSCLE PROTEINS

Sulfur Amino Acids in *Animal Muscle Proteins*

Calculated to 16.0 gm. N.

ANIMAL	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Beef Muscle	Hopkins, Beach	Beach	58	14.8	1.1	1.1	3.5
Beef Muscle	Hopkins, Beach	Beach	59		1.1	1.3	3.2
Beef Muscle	Sullivan, McCarthy	Beach	59		1.1	0.9	3.1
Beef Muscle	Folin	Jones	342	(16.0)		1.6	
Beef Muscle	Sullivan	Pottinger	527	(16.0)		1.3	
Beef Muscle	Folin, Calculated	unpublished		16.1	1.0	1.1	3.3
Veal Muscle	Hopkins, Beach	Beach	59		1.1	1.3	3.3
Veal Muscle	Sullivan, McCarthy	Beach	59			1.0	3.6
Lamb Muscle	Hopkins, Beach	Beach	59		1.1	1.4	3.1
Lamb Muscle	Sullivan, McCarthy	Beach	59			1.0	3.3
Pork Muscle	Hopkins, Beach	Beach	59		1.0	1.1	3.4
Pork Muscle	Sullivan, McCarthy	Beach	59			1.0	3.2
Chicken Muscle	Hopkins, Beach	Beach	59		1.04	1.3	3.2
Chicken Muscle	Sullivan, McCarthy	Beach	59			0.9	3.5
Chicken Myosin	Folin, Baernstein	Bailey	44	16.6	1.02	0.7	3.2
Turtle Muscle	Hopkins, Beach	Beach	59		1.07	1.3	3.0
Turtle Muscle	Sullivan, McCarthy	Beach	59			0.6	4.1
Frog Muscle	Hopkins, Beach	Beach	59		1.1	1.3	3.2
Rabbit-Myogen	Folin, Baernstein	Bailey	44	16.6	1.24	1.9	2.7
Rabbit-Myogen	Sullivan	Bailey	44	16.6		1.8	
Rabbit-Myosin	Folin, Baernstein	Bailey	44	16.7	1.05	0.7	3.3
Rabbit-Myosin	Folin, Baernstein	Sharp	575	16.8		0.7	3.2
Dog-Myosin	Folin, Baernstein	Bailey	44	16.6	1.08	0.8	3.3
Rat Muscle	Folin	Lee	411		1.0	1.1	
Mean with 2×S.E.					1.1	1.1±0.1	3.3±0.1

MUSCLE PROTEINS

Sulfur Amino Acids in *Fish and Crustacean Muscle Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Cod	Sullivan, Isolation	Abderhalden	24	13.6	0.15*	0.7	0.4*
Cod	Sullivan	Pottinger	527	(16.0)		1.4	
Cod	Hopkins, McCarthy	Beach	59		1.19*	1.2*	3.7*
Halibut	Gasometric, Baernstein	Baernstein	41	(16.0)	1.16	1.9	4.0
Halibut	Sullivan	Hess	291	(16.0)		0.8	
Halibut	Sullivan	Pottinger	527	(16.0)		1.5	
Croaker	Sullivan	Pottinger	527	(16.0)		1.2	
Haddock	Sullivan	Pottinger	527	(16.0)		1.2	
Haddock Meal	Folin	Pottinger	526			1.1	
Mackeral	Sullivan	Pottinger	527	(16.0)		1.3	
Mullet	Sullivan	Pottinger	527	(16.0)		1.3	
Menhaden Meal	Fleming, Calculated	unpublished		11.6	0.9	1.0	3
Red Snapper	Sullivan	Pottinger	527	(16.0)		1.3	
Salmon	Sullivan	Pottinger	527	(16.0)		1.3	
Salmon	Hopkins, Beach	Beach	59		1.22*	1.2*	3.2*
Shad	Sullivan	Pottinger	527	(16.0)		1.2	
Fish-Myosin	Folin, Baernstein	Bailey	44	16.6	1.17	0.9	3.5
Fish	Folin	Jones	342	(16.0)		1.3	
Shrimp	Gasometric, Baernstein	Baernstein	41	(16.0)	1.25	1.6	3.4
Shrimp	Sullivan	Hess	291	(16.0)		0.9	
Shrimp	Hopkins, Beach	Beach	59		1.16*	1.1*	3.0*
Shrimp	Sullivan	Pottinger	527	(16.0)		1.3	
Lobster	Folin, Baernstein	Bailey	44	16.1	1.17	0.9	3.3
Mean with 2 ×S.E.					1.13	1.2 ± .1	3.4
* Omitted from mean							

MUSCLE PROTEINS

The essential similarity in the cystine and methionine yielded by animal, fish, and crustacean muscle proteins is of interest both to the comparative biochemist and to the nutritionist.

PLANT PROTEINS

Sulfur Amino Acids in the Proteins of *Autotropic Organisms*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		CYSTINE	METHIONINE
				gm.	gm.
Phormidium	Schulz, Calculated	Mazur	442	0.0	3.4
Ulva	Schulz, Calculated	Mazur	442	2.7	0.0
Laminaria	Schulz, Calculated	Mazur	442	4.7	0.0
Sargassum	Schulz, Calculated	Mazur	442	4.0	0.0
Chondrus (Irish Moss)	Schulz, Calculated	Mazur	442	2.0	0.0
Osmunda (Fern)	Schulz, Calculated	Mazur	442	0.8	0.0
Gloeotrichia	Schulz, Calculated	Mazur	443	0.0	24.3
Macrocystis	Schulz, Calculated	Mazur	443	0.8	28.9
Lessoniopsis	Schulz, Calculated	Mazur	443	1.5	5.8
Fucus	Schulz, Calculated	Mazur	443	2.1	9.4
Cystoseira	Schulz, Calculated	Mazur	443	3.0	10.5
Egregia	Schulz, Calculated	Mazur	443	0.8	4.3
Caulerpa	Schulz, Calculated	Mazur	443	0.8	2.7
Codium	Schulz, Calculated	Mazur	443	0.7	3.9
Diatoms	Schulz, Calculated	Mazur	443	0.8	4.1
Pteridium (Fern)	Schulz, Baernstein	Lugg	655	1.6*	2.8*

* "Best Values."

PLANT PROTEINS

Sulfur Amino Acids in *Biologically Active Substances*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE
				gm.	gm.	gm.
Yellow Enzyme	Sullivan, Folin	Kuhn	393	16.3	0.47	0.5
Yellow Enzyme	Folin	Kuhn	394	16.3		0.4
Cottonseed Allergen	Sullivan	Spies	585	19.8	1.86	3.6
Cottonseed Allergen	Sullivan	Spies	585	20.2	1.77	3.9
Cottonseed Allergen	Sullivan	Spies	585	11.6	2.81	5.9
Ricin		Karrer	355	(17.0)		1.0
Crystalline Wheat Peptide		Balls	48	17.4	4.1	15.2

PLANT PROTEINS

Sulfur Amino Acids in *Corn Proteins* other than Zein

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE	NITROGEN	SULFUR	CYSTINE	METH-IONINE
			per cent	gm.	gm.	gm.
Corn, White, whole	Folin	unpublished		1.4	1.5	
Corn, White, whole	Sullivan	Csonka 181			0.9	
Corn, Yellow, whole	Folin	unpublished		1.7	1.2	
Corn, Yellow, whole	Sullivan	Csonka 181			0.8	
Gluten, White	Folin, Calculated	unpublished	10.9	1.3	1.5	4
Gluten, White	Fleming-Vassel	unpublished	10.9		0.7	
Gluten, Yellow	Folin, Calculated	unpublished	12.7	1.5	1.5	5
Gluten, Yellow	McCarthy	unpublished	11.6			5.5
Glutelin	Sullivan	Csonka 177	(16.0)		0.5	
Germ, White	Folin, Calculated	unpublished	11.8	1.1	1.1	3
Germ, White	Fleming-Vassel	unpublished			0.6	
Germ, Yellow	Folin, McCarthy	unpublished	12.8	1.0	1.8	1.6
Albumins	Folin, Calculated	unpublished	12.6	0.9	0.5	2
Zein Residue	Folin, McCarthy	unpublished	10.8		2.2	4.8
Zein Residue	Fleming-Vassel	unpublished	10.8		1.3	

PLANT PROTEINS

Sulfur Amino Acids in *Edestin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
			per cent	gm.	gm.	gm.
Gasometric	Baernstein	39	(18.6)		1.5	
Folin	Baernstein	39	(18.6)		1.2	
Mörner-Okuda	Baernstein	39	(18.6)		1.0	
Gasometric, Baernstein	Baernstein	41	(18.6)	0.85	1.5	1.8
Baernstein-Iodide	Baernstein	42	(18.6)			2.0
Baernstein-Titration	Baernstein	42	(18.6)			1.9
Baernstein-Iodide	Baernstein	42	(18.6)			1.2
Baernstein-Titration	Baernstein	42	(18.6)			1.2
Sullivan, Baernstein	Bailey	43	18.4	0.81	1.1	2.0
Folin	Bailey	43	18.4		1.2	
Hopkins, Baernstein	Beach	55	17.1		1.0	2.3
Hopkins, Beach	Beach	58	18.5	0.82	1.0	2.0
Folin	Folin	231	(18.6)		0.7	
Folin	Folin	234	(18.6)		1.2	
Sullivan	Gordon	261			0.5	
Sullivan	Hess	291	(18.6)		1.1	
Sullivan, Baernstein	Lugg	431	18.5		1.2	2.0
Baernstein	Lugg	432	18.5		1.3	2.1
Folin	Marenzi	436	(18.6)		1.2	
McCarthy	McCarthy	444	18.4			2.1
Brdicka	Stern	590	(18.6)		1.0	
Hopkins-Graff	Stern	590	(18.6)		1.0	
Sullivan	Sullivan	596	(18.6)		1.0	
Folin	Tompsett	615	(18.6)		1.3	
Fleming-Vassel	Vassel	641	(18.6)		1.0	
Hopkins-Vickery	Vickery	652	(18.6)		1.1	
Schulz	Zahnd	695	(18.6)		1.1	
Mean with 2 ×S.E.				0.83	1.1 ± .3	1.9 ± .2

PLANT PROTEINS

Sulfur Amino Acids in *Gliadin*

Calculated to 16.0 gm. N.

METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
			per cent	gm.	gm.	gm.
Gasometric, Baernstein	Baernstein	41	(17.7)	0.9	2.5	2.7
Sullivan, Baernstein	Bailey	43	17.1	1.12	2.3	1.5
Folin	Bailey	43	17.1		2.4	
Folin	Folin	231	(17.1)		2.2	
Folin	Folin	234	(17.1)		2.1	
Hopkins-Graff	Graff	264			2.1	
Sullivan	Hess	291	(17.1)		2.0	
Folin	Jones	342	(17.1)		1.6	
Folin	Marenzi	436	(17.1)		2.1	
Sullivan	Sullivan	596	(17.1)		2.1	
Hopkins	Vickery	652	(17.1)		1.9	
Mean with 2 ×S.E.				1.0	2.1 ± .2	2.1

PLANT PROTEINS

Sulfur Amino Acids in *Grass Proteins*

Calculated to 16.0 gm. N.

GRASS	METHOD[*]	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Cocksfoot	Sullivan, Baernstein	Bailey	43	13.4	1.2	0.2*	
Cocksfoot	Sullivan, Baernstein	Lugg	431	14.1		2.0	2.2
Cocksfoot	Baernstein	Lugg	432	12.9		2.1	2.4
Cocksfoot	Schulz, Baernstein	Lugg	433			2.2	2.1
Cocksfoot	Sullivan	Pollard	524			0.3*	
Canary	Sullivan, Baernstein	Lugg	431	15.4		2.0	2.1
Canary	Baernstein	Lugg	432	15.4		2.2	2.5
Rye	Baernstein	Lugg	432	13.5		2.3	2.7
Rye	Schulz, Baernstein	Lugg	433			2.1	2.3
Rye	Sullivan	Pollard	524			0.5*	
Fescue	Baernstein	Lugg	432	14.3		2.2	2.8
Lucerne	Sullivan, Baernstein	Lugg	431	13.9		1.5	2.2
Lucerne	Baernstein	Lugg	432	14.0		1.7	2.2
Salt-bush	Sullivan, Baernstein	Lugg	431	12.1		1.6	2.6
Salt-bush	Baernstein	Lugg	432	12.1		2.0	2.6
Dogtail	Baernstein	Lugg	432	14.1		2.3	2.7
Mean with 2×S.E.					1.2	2.0±.1	2.4±.1
* Omitted from Mean							

PLANT PROTEINS

Sulfur Amino Acids in *Leaf Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Alfalfa	Folin	unpublished		10.6	1.3	1.4	
Clover-white	Baernstein	Lugg	432	13.1		1.7	2.3
Clover-red	Baernstein	Lugg	432	12.8		1.7	2.2
Clover-red	Sullivan	Pollard	524			0.2*	
Runner bean	Baernstein	Lugg	432	13.3		1.8	2.1
Turnip tops	Baernstein	Lugg	432	13.0		1.9	2.8
Beet tops	Baernstein	Lugg	433			2.0	2.0
Mean with 2×S.E.						1.8±.2	2.3±.3
* Omitted from mean							

PLANT PROTEINS

Sulfur Amino Acids in *Miscellaneous* Plant Proteins

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	SUL-FUR	CYS-TINE	METH-IONINE
				per cent	gm.	gm.	gm.
Cottonseed Globulin	Baernstein	Fontaine	238	17.2	0.70	1.1	2.3
Cottonseed Globulin	Folin	Jones	342	(17.2)		1.0	
Cottonseed Globulin	Schulz	Zahnd	695	(17.2)		1.0	
Cottonseed Meal	Folin, McCarthy	unpublished		10.9		2.0	1.6
Linseed Meal	Folin, Calculated	unpublished			1.1	1.9	3
Peanut Meal	Folin, McCarthy	unpublished		10.4		1.6	0.9
Arachin	Gasometric, Baernstein	Baernstein	41	(18.0)	0.37	1.2	0.5
Arachin	Hopkins, Beach	Beach	58	17.7	0.43	0.8	0.5
Arachin	Baernstein	Bennett	63	(18.0)			0.7
Arachin	Folin	unpublished		17.0		0.9	
Arachin	Baernstein	Brown	133	18.0	0.46	1.3	0.6
Arachin	Hopkins-Graff	Graff	264			0.8	
Arachin	Sullivan	Hess	291	(18.0)		1.2	
Arachin	Sullivan, McCarthy	Hess	295	(18.0)		1.1	0.4
Arachin	Folin	Jones	342	(18.0)		1.0	
Arachin	Sullivan	Sullivan	596	(18.0)		0.8	
Arachin	Mean with 2×S.E.				0.42	1.0±.2	0.5
Conarachin	Baernstein	Brown	133	18.0	1.08	2.6	1.9
Conarachin	Folin	Jones	342	(18.0)		2.7	
Soybean Raw	McCarthy-Sullivan	Almquist	29				1.9
Soybean Meal	McCarthy-Sullivan	Almquist	29				2.0
Soybean Meal	Folin, McCarthy	unpublished				1.3	1.6
Soybean Meal	Sullivan	Hamilton	277			0.6[a]	
Soybean Meal	Sullivan	Hamilton	277			1.4[b]	
Flaxseed Meal	Folin, McCarthy	unpublished				1.9	2.3
Glycinin	Gasometric, Baernstein	Baernstein	41	(17.5)	0.77	1.5	1.7
Glycinin	Folin	Jones	342	(17.5)		1.1	
Hordein	Gasometric, Baernstein	Baernstein	41	(17.2)	0.84	1.6	2.1
Rubber Latex	Baernstein	Tristram	620	15.0		1.0	1.1
Rice-Glutelin	Sullivan	Csonka	177			1.2	
Rice-Bran	Sullivan	Kik	364			1.0	
Rice-Whole	Sullivan	Kik	364			1.3[c]	
Rice-Cereal	McCarthy-Sullivan	unpublished					3.1
Oats-Whole	Sullivan	Csonka	182			0.7	
Oats-Cereal	Fleming, McCarthy	unpublished				1.8	2.3

[a] Ohio 13–177 lowest of a number of samples analyzed
[b] Mansoy highest of a number of samples analyzed
[c] Average of 7 samples which varied from 1.1 to 1.5 per cent of cystine.

PLANT PROTEINS

Sulfur Amino Acids in *Viruses*

Calculated to 16.0 gm. N.

PROTEIN	METHOD	REFERENCE	NITROGEN	CYSTINE	HYDROLYSIS
			gm.	gm.	
Tobacco Mosaic[b]	Sullivan	Hess 294	(16.0)	0.5	HCl
Tobacco Mosaic	Sullivan	Hess 294	(16.0)	0.5	HCl-HCOOH
Tobacco Mosaic	Sullivan	Hess 294	(16.0)	0.7	H_2SO_4 under N
Tobacco Mosaic	Sullivan	Hess 294	(16.0)	0.6	HCl-$TiCl_3$
Tobacco Mosaic	Sullivan	Hess 294	(16.0)	0.7	HI
Tobacco Mosaic	Sullivan	Hess 294	(16.0)	0.7*	HI-HCOOH
Tobacco Mosaic	Sullivan	Ross 555	15.9	0.5	HCl and/or HCOOH
Tobacco Mosaic	Folin	Ross 555	15.9	0.3	HCl and/or HCOOH
Tobacco Mosaic[a]	Baernstein	Ross 555	15.9	0.7*	

[a] Methionine 0.0?
[b] Sulfur 0.24 per cent
* Best value

PLANT PROTEINS

Sulfur Amino Acids in *Wheat Proteins* other than Gliadin

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH-IONINE
				per cent	gm.	gm.	gm.
Wheat-Hard	Sullivan	Csonka	179			1.3	
Wheat-Hard	Sullivan	Csonka	179		(	1.1	
Wheat-Soft	Sullivan	Csonka	179			1.1	
Wheat-Whole	Folin	unpublished			1.4	1.8	
Wheat-Whole	Fleming-Vassel	unpublished				1.0	
Flour	Folin, Calculated	unpublished		12.8	1.1	1.9	3
Flour-Hard	Sullivan	Csonka	180			1.6	
Flour-Hard	Sullivan	Csonka	180			1.7	
Gluten	Sullivan	Padoa	508	?		1.4	
Glutelin	Sullivan	Csonka	177	(16.0)		1.3	
Glutenin	Folin	Jones	342	(16.0)		1.6	
Glutenin	Folin	Marenzi	436	(16.0)		0.7	
Germ	Folin, Calculated	unpublished			0.5	0.8	2
Germ	Fleming-Vassel	unpublished				0.4	
Bran	Sullivan	Csonka	180			0.5	
Bran-Albumin	Folin	Jones	342	(16.0)		3.3	
Bran-Globulin	Sullivan	Sullivan	596	(16.0)		0.5	
Shorts	Sullivan	Csonka	180			0.6	

PLANT PROTEINS

Sulfur Amino Acids in *Yeast Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITROGEN	SULFUR	CYSTINE	METH-IONINE
			per cent	gm.	gm.	gm.
Brewer's	Folin, Calculated	unpublished	9.7	0.8	1.0	2
Brewer's	Folin	unpublished	9.7		1.5	
Brewer's	Folin, Calculated	unpublished	14.4	0.7	2.2	1-2
Brewer's	Folin, Calculated	unpublished		0.9	1.4	2
Brewer's	Folin	unpublished			1.5	
Brewer's	Folin, Calculated	unpublished		0.8	0.8	2-3
Brewer's	Sullivan	Csonka 178		0.6	0.5	
Brewer's	Sullivan	Prunty 530	(8.0)		1.8	
Brewer's	Sullivan	Prunty 530	(8.0)		1.8	
Baker's	Folin, Calculated	unpublished		0.9	0.7	
Baker's	Sullivan	Csonka 178		0.8	0.7	3
Baker's	Sullivan	Prunty 530	(8.0)		1.3	
Steep Water	Folin, Calculated	unpublished		1.3	2.2	3
Mean with 2×S.E.				0.9	1.3±.3	2-3

PLANT PROTEINS

Sulfur Amino Acids in *Zein*

Calculated to 16.0 gm. N.

METHOD	REFERENCE	NITROGEN	SULFUR	CYSTINE	METH-IONINE
		per cent	gm.	gm.	gm.
Baernstein-Iodide	Baernstein 42	(16.1)			2.5
Baernstein-Titration	Baernstein 42	(16.1)			2.4
	Blumenthal 111	(16.1)	0.52		
Folin	Folin 231	(16.1)		0.5	
Folin	Folin 234	(16.1)		1.0	
Sullivan	Hess 291	(16.1)		0.9	
Folin	Jones 342	(16.1)		0.9	
Brdicka	Laine 398	(16.1)		0.8	
Sullivan	Sullivan 598	(16.1)		0.8	
Hopkins	Vickery 652	(16.1)		0.9	
Baernstein	Virtanen 663	14.5			2.2
Schulz	Zahnd 695	(16.1)		0.8	
Average			0.5-0.6	0.8	2.4

PLANT PROTEINS

Autotropic Organisms: The apparent absence of methionine, calculated to be sure from the noncystine organic sulfur, from the total tissue proteins of certain marine organisms, is even less surprising than the exceedingly high values reported for this amino acid in other autotropic organisms. (In contrast *cf.* 433A.)

Biologically Active Substances: The presence of 15 per cent of cystine in a protein or polypeptide from wheat bran is of interest when one recalls the large quantity of this amino acid usually present in eukeratins.

Corn Proteins: The relatively large quantities of methionine in corn gluten has not been generally recognized and may be an important factor in its value as a feedstuff.

Grass and Leaf Proteins: The similarity in cystine and methionine content of all the grass and leaf proteins analyzed so far is in marked contrast to the large differences claimed to be present in the autotropic organisms. However, Lugg finds the autotropic plants similar in amino acid composition to the higher plants.

Peanut Proteins: These are unusually low in methionine, a fact long indicated by the analyses of arachin by Baernstein and the feeding experiments of Beach.

Soybean Proteins: Variations of over 100 per cent in the cystine content of different varieties of soybeans are of special interest in view of the widespread tendency to use soybeans, irrespective of amino acid content, as "meat substitutes."

Wheat Proteins: Wheat gluten does not appear to be as well supplied with methionine as corn gluten, although further studies are necessary. The relatively high cystine content in bran albumin reported by Jones is interesting in the light of Balls' recent isolation of a polypeptide from wheat bran which contains 15 per cent of cystine.

TISSUE PROTEINS

Sulfur Amino Acids in *Animal Tissue Proteins*

Calculated to 16.0 gm. N.

TISSUE	METHOD	REFERENCE		NITROGEN	SULFUR	CYSTINE	METH- IONINE
				per cent	gm.	gm.	gm.
Kidney-Human	Folin	Block	105	13.3		1.6	
Kidney-Rat	Folin	Lee	411		1.1	1.8	
Kidney-Beef	Folin, Calculated	unpublished		15.6	1.0	1.5	2.8
Kidney-Beef	Sullivan, Beach	Beach	59		1.1	1.2	2.7
Spleen-Beef	Folin, Calculated	unpublished		15.7	0.9	1.3	2.6
Thymus-Beef	Folin, Calculated	unpublished		15.4	0.8	1.1	2.4
Intestine-Beef	Folin, Calculated	unpublished		15.4	0.8	1.2	2.2
Lung-Beef	Folin, Calculated	unpublished		15.3	0.9	1.4	2.5
Lung-Beef	Hopkins, McCarthy	Beach	59		1.0	1.5	2.5
Heart-Beef	Folin, Calculated	unpublished		14.8	1.1	1.2	3.6
Heart-Beef	Sullivan, McCarthy	Beach	59		1.1	1.2	3.2
Bladder-Beef	Folin, Calculated	unpublished		15.9	0.9	1.1	2.8
Pancreas-Beef	Folin, Calculated	unpublished		15.5	0.9	1.5	2.3
Ovaries-Beef	Folin, Calculated	unpublished		15.8	1.0	1.2	3.2
Testes-Beef	Folin, Calculated	unpublished		15.4	0.9	1.2	2.7
Salivary-Beef	Folin, Calculated	unpublished		15.7	0.9	0.9	3.1
Adrenals-Beef	Folin	unpublished		15.8		1.7	
Stomach-Beef	Hopkins, Beach	Beach	59		1.0	1.0	2.0
Pooled tissue, Mean with 2×S.E.					0.9±.1	1.3±.1	3

TISSUE PROTEINS

Animal tissue proteins, exclusive of supporting, connective, or protective tissues, appear to have from one to one and a half per cent of cystine and approximately 3 per cent of methionine.

THE β-HYDROXY AMINO ACIDS

SERINE AND THREONINE

	Serine	Threonine
Empirical Formula	$C_3H_7O_3N$	$C_4H_9O_3N$
Optical Form	*l*	*d*
Molecular Weight	105.06	119.08
Carbon	34.27	40.31
Hydrogen	6.72	7.62
Nitrogen	13.33	11.74
Oxygen	45.68	40.31
Melting Point	228° (decomp.)	251–3° (cor.)

PART I

HYDROLYSIS

SERINE and threonine, like cysteine, are readily destroyed by boiling with dilute alkalies, but appear to be quite stable to hydrolysis with dilute acids, even in the presence of carbohydrates. However, Borchers, Totter, and Berg (115) found that long heating and high concentrations of sulfuric acid would also result in destruction of threonine. Our own experience has suggested that the quantity of threonine found in a protein hydrolysate is somewhat dependent on the time and temperature of hydrolysis. This observation requires further study.

Nicolet, Shinn and Saidel (479) have reported that the phosphorylated serine groups in casein, vitellin, etc., undergo destruction during acid hydrolysis. They, therefore, suggest warming the phosphoprotein at 37° for 24 hours with an excess of 0.25 N NaOH to liberate the phosphoric acid groups and then hydrolyze with acid as usual. "To serine as determined in this hydrolysate is added a (small) correction for non-phosphorylated serine destroyed by alkali, and one molecular equivalent for all phosphate liberated." This correction results in considerably higher serine values than would be obtained directly. Thus, vitellin (N = 15.26 per cent) yielded 7.70 per cent serine, uncorrected, 9.0 per cent corrected, while casein (N = 15.80 per cent) gave 5.5 per cent of serine, corrected 7.4 per cent.

Even though these procedures may not yield the absolute quantities of threonine and serine present in the intact protein due to hydrolytic losses and to the possibility of incomplete oxidation; "The results," say Martin and Synge (438), "are at, least, of comparative significance, and set lower limits to the amounts of the amino-acids in question that are present."

PART II

THE DETERMINATION OF THREONINE

Historical: In 1939, Block and Bolling (99, 100) showed that of all the amino acids commonly occurring in a protein hydrolysate, threonine alone yielded acetaldehyde and in the expected (calculated) amount. The following reaction mechanism, according to Criegee, Kraft, and Rank (173), was suggested:

$$-\overset{|}{C}-OH \atop -\overset{|}{C}-OH \underset{|}{} \quad +Pb(CH_3COO)_4 \rightarrow \quad -\overset{|}{C}-O-Pb(CH_3COO)_3 \atop -\overset{|}{C}-OH \quad \rightarrow \quad \genfrac{}{}{0pt}{}{-\overset{|}{C}-O}{-\overset{|}{C}-O} \!\! \diagdown \!\! Pb(CH_3COO)_2$$

$$\rightarrow -\overset{|}{C}=O + -\overset{|}{C}=O + Pb(CH_3COO)_2$$
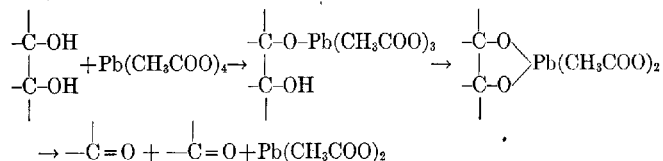
The acetaldehyde thus formed was determined by aerating it into p-hydroxydiphenyl in concentrated H_2SO_4 according to Eegriwe (206). The high degree of specificity for CH_3CHO of this reagent has been shown by Miller and Muntz (450) and by Barker and Summerson (49).

1. OXIDATION TO ACETALDEHYDE WITH SPECIFIC OXIDANTS

A. The Lead Tetraacetate Method of Block and Bolling (99, 100)

Principle: Threonine is oxidized by lead tetraacetate to yield acetaldehyde which is aerated into concentrated H_2SO_4 containing p-hydroxydiphenyl. A stable red-purple colored compound is formed.

Reagents: Lead Tetraacetate (Fieser, 218). Mechanically stir a mixture of 500 ml. of glacial acetic acid and 400 ml. of acetic anhydride. Warm to 55° and add 650 gm. of powdered, dried Pb_3O_4 (red lead) in 20 gm. portions. Wait for the red color to disappear before adding the next portion. Maintain the temperature between 50° and 80°C. Cool the reaction mixture, filter and wash the lead tetraacetate. Recrystallize the moist salt from boiling acetic acid which is "dried" by the addition of a small quantity of acetic anhydride. Yield 350 gm.

Lead Tetraacetate (Oesper and Deasy, 481). Mechanically stir 600 ml. of glacial acetic acid and 150 ml. of acetic anhydride at

65°. Bubble a steam of chlorine gas through the mixture. Then add 120 gm. of Pb_3O_4, which has previously been dried at 150° for 1 to 2 hours, in five equal portions, waiting between each portion for the red color to disappear. Maintain the temperature between 65° and 80°. Decant the hot solution through a heated filter and then cool the filtrate. Remove and wash the lead tetraacetate with acetic acid-acetic anhydride. This material should be 90 per cent $Pb(CH_3COO)_4$.

Reextract the original residue with warm (70–80°) glacial acetic acid to obtain a second crop.

$$Pb_3O_4 + 8CH_3COOH \rightarrow Pb(CH_3COO)_4 + 2Pb(CH_3COO)_2 + 4H_2O$$
$$2Pb(CH_3COO)_2 + Cl_2 \rightarrow Pb(CH_3COO)_4 + PbCl_2$$
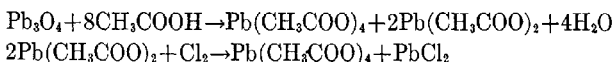
p-Hydroxydiphenyl (Miller and Muntz, 450). Dissolve commercial p-hydroxydiphenyl in hot purified acetone and crystallize it out from the cold solution by the addition of water. Repeat this process. Crystallize the compound a third time from the minimum quantity of hot. acetone without the addition of water. Dry the p-hydroxydiphenyl at room temperature in a dark place protected from dust.

Glacial Acetic Acid. Purify by refluxing for 7 hours with 0.5 to 1.0 per cent of $K_2Cr_2O_7$, and then distilling in an all glass apparatus.

Apparatus: Six 20×2.5 cm. Pyrex test tubes, connected with short lengths of pressure tubing, are arranged in series with inlet and outlet tubes according to the usual gas adsorption train and with 29/42 interchangeable ground glass joints.

Tube 1 contains 20 ml. of concentrated H_2SO_4 to wash and dry the incoming air.

Tube 2 is empty.

Tube 3, the oxidizing tube, contains 25 ml. of purified glacial acetic acid, less than 10 mg. of amino acid solution, and 1 gm. of lead tetraacetate. The oxidizing tube is maintained at 30°C.

Tube 4 is empty, but is kept in a 4° bath to condense the acetic acid vapors.

Tube 5 contains pellets of NaOH to trap any residual acetic acid.

Tube 6, the color tube, which contains 15 ml. of concentrated H_2SO_4 and 50 to 100 mg. of purified p-hydroxydiphenyl, is kept in an ice bath at 0° throughout the aeration.

Method: 1. Hydrolysis. 10 to 15 mg. of protein are hydrolyzed with 8 N H_2SO_4 under reflux in an oil bath at 115 to 125° over night. No caprylic alcohol should be used. The neutralized hydrolysate is evaporated to a thin syrup either *in vacuo* or on the steam bath to remove any trace of alcohol, or other volatile substances which

would yield CH_3CHO. The residue is dissolved in water or in purified glacial acetic acid.

2. Oxidation. An aliquot of the solution, which contains 0.5 to 1.0 mg. of protein, is pipetted into Tube 3 and the oxidation is allowed to take place at 30° for 1 hour, the CH_3CHO is aerated through Tubes 4 and 5 into Tube 6 at a fairly rapid rate. It is advisable to use a gas flowmeter on the intake side so that, once standard conditions have been established, the conditions of aeration can be kept approximately constant.

3. Color Development. At the end of the aeration, the color tube, which still contains considerable undissolved p-hydroxydiphenyl, is placed in a boiling water bath for exactly 2 minutes, in order to dissolve the excess hydroxydiphenyl, and then cooled in an ice bath. An alternate procedure is to transfer the contents of Tube 6 into a reading tube and allow the p-hydroxydiphenyl to rise to the top so that the clear solution can be read in the usual photoelectric colorimeter, light filter 560 mu.

Calibration curves over the range 0.03 to 0.07 mg. of threonine should be prepared.

Comment: Because maximum color will not be formed if the rate of aeration is either too fast or too slow, it is advisable to connect two series of tubes to permit the determinations of acetaldehyde yielded by the protein and by a threonine standard under closely comparable conditions. The order of the unknown and standard solutions should be reversed in the train with each successive run.

The color is very stable, lasting at least 24 hours. The method permits the determination of as little as 0.004 mg. of threonine. Both threonine and lactic acid yield the calculated quantities of CH_3CHO, identified as the dinitrophenylhydrazone (M.P. 159–161°, Bial and Weiss, 74).

Borchers, Totter and Berg (115) confirmed the finding of Block and Bolling (100) that $d(-)$threonine yields 100 per cent of the expected amount of acetaldehyde, but they found that allothreonine yielded only approximately one half of that expected. They also found that alanine yielded 2 per cent of the theoretical quantity of CH_3CHO.

Borchers, Totter and Berg (115) reported that the tetraacetate method could be carried out with an accuracy of less than 5 per cent, but they point out that it is too finicky for casual application.

B. The Periodate Method of Shinn and Nicolet (578)

Principle: Like lead tetraacetate, periodic acid will oxidize 1,2 diglycols and related compounds to yield the corresponding

aldehydes (Malaprade, 435). Thus threonine yields CH_3CHO and serine $HCHO$. Shinn and Nicolet (578) did not attempt to use a specific method for the determination of acetaldehyde, but made the crucial observation that in the presence of excess amino groups, the $HCHO$ formed from serine, is completely retained in the oxidizing mixture and only CH_3CHO is aerated into bisulfite.

Apparatus: A three tube gas adsorption train similar to that described in A.

Reagents: 0.1 N Sodium Arsenite, Na_2HAsO_3, should contain 20 gm. of $NaHCO_3$ per liter.

0.5 M Periodic Acid, H_5IO_6.

2 per cent Sodium Bisulfite, $NaHSO_3$ containing 19 gm. of metabisulfite per liter.

0.1 N Iodine: 13 gm. of I_2 and 30 gm. of KI are dissolved in 250 ml. of water and the solution is diluted to 1 liter. This solution is standardized with 0.1 N $Na_2S_2O_3$ (24.82 gm. of $Na_2S_2O_3 \cdot 5H_2O$ per liter).

1 per cent starch in water.

$CHCl_3$ must not be used as a preservative.

Method: 1. Hydrolysis. A small sample of protein is hydrolyzed with 1:1 HCl for 24 hours. The excess acid is neutralized and the solution is diluted to volume.

2. Oxidation. An aliquot of the hydrolysate containing 3 to 10 mg. of threonine in 5 ml. of solution is placed in Tube 1 together with 1 drop of Nujol, 5 ml. of M $NaHCO_3$ and 10 ml. of Na_2AsO_3 solution. The tubes are connected with a CO_2 supply and the gas is passed in to mix the contents. Then 1 to 2 ml. of 0.5 M H_5IO_6 are added, and the CH_3CHO is aerated into the two receiving tubes, the first of which contains 5 ml. and the second 3 ml. of 2 per cent $NaHSO_3$, diluted in each case to 25 ml. The aeration is continued for 1 hour at the rate of 1 liter of gas per minute. The oxidizing solution is set aside for the determination of serine (*cf.* Part III, Section A of this chapter).

3. Determination. The contents of Tubes 2 and 3 are mixed and the CH_3CHO is determined by titration according to Peters and Van Slyke (516).

The free $NaHSO_3$ is removed with 0.1 N iodine using 1 ml. of starch solution as the internal indicator. The excess I_2 is removed with 1 drop of 0.1 N $Na_2S_2O_3$, then 0.002 N iodine is added carefully to the point where 1 drop produces a faint color, but not blue.

The combined $NaHSO_3$ is then liberated by the addition of a small amount of saturated $NaHCO_3$ or Na_2HPO_4 (Winnick, 683) and the free $NaHSO_3$ is titrated with 0.02 N iodine. The end point

is tested with a little NaHSO₃. The blue color should remain 15 seconds or longer.

$$1 \text{ ml. of } 0.02 \text{ N I}_2 \leftrightharpoons 1.19 \text{ mg. of threonine.}$$

Comment: Borchers, Totter and Berg (115) found that *d*(-)threonine, on periodate oxidation, yielded 96 per cent of the expected quantity of acetaldehyde while Martin and Synge (438) reported that the natural amino acid yielded only approximately 70 per cent of the theoretical quantity. The latter investigators agreed with the former, that periodate oxidation of synthetic *dl*-threonine gave 100 per cent of the calculated amount of acetaldehyde.

Martin and Synge (438) recommend that the protein hydrolysate be concentrated twice *in vacuo* to remove alcohol, etc.

Nicolet (478) has changed the above method to permit the determination of threonine in the presence of sugars, including methylpentoses. The oxidation is modified by adding the HIO₄ *before* rather than after the introduction of the arsenite. One aliquot of the hydrolysate is made alkaline to litmus with NaHCO₃ and an excess of NaHCO₃ is added to neutralize the acetic acid. Then 3 mols of acetic anhydride, calculated from the nitrogen of the aliquot, in 5 to 10 volumes of benzene, are added with stirring, in 3 equal portions. The benzene is removed by aeration. Threonine is calculated from the difference in total CH₃CHO and that found after acetylation.

C. Winnick's Microadaptation of the Shinn Method (685)

Apparatus: Conway microdiffusion cups (169, 170, 276).

Method: 1. Hydrolysis. 500 mg. of protein are hydrolyzed with 20 ml. of 3 N HCl for 24 hours. The hydrolysate is neutralized to *p*H 7.0 and diluted so that 2 ml. contains approximately 0.2 to 0.5 mg. of threonine.

2. Oxidation and Diffusion. 1.5 ml. of 0.25 M NaHSO₃ are placed in the central chamber of the Conway cup (169). In the outer chamber, 3 ml. of unknown, 1 ml. of 0.1 M K₃PO₄ (1 ml. of K₃PO₄ should neutralize 1 ml. of 0.2 M HIO₄ to *p*H 7.0), and 1 ml. of 0.2 M HIO₄ are introduced in order. The vessel is covered, rotated to mix the solutions, and the oxidation and diffusion are allowed to take place at room temperature for 4 to 5 hours.

3. Determination. A drop of starch solution is added to the inner chamber and the free NaHSO₃ is removed with N iodine to a permanent light purple color. If the end point is passed, the solution is decolorized with a drop of NaHSO₃ and the proper quantity of iodine is added.

The bound NaHSO₃ is then liberated by stirring in 200 to 400 mg. of powdered Na₂HPO₄. The freed NaHSO₃ is titrated with 0.005 N iodine (*cf.* 683, 684, 685).

1 ml. of 0.005 N·I₂ ≈ 0.298 mg. of threonine.

D. The Periodate-p-Hydroxydiphenyl Method
(Block and Bolling, 104)

Apparatus: Two groups of three 20×2.5 cm. Pyrex test tubes connected in series as gas washing trains, are used.

The first tube in each series contains 20 ml. of concentrated H_2SO_4 or 2 per cent NaHSO₃ to wash the incoming air. The second tube is for oxidizing and the third tube contains 15 ml. of concentrated H_2SO_4 and approximately 100 mg. of purified p-hydroxydiphenyl (*cf.* A).

Method: An aliquot of the neutralized protein hydrolysate (*cf.* Section A, *Method*) containing 0.03 to 0.06 mg. of threonine is pipetted into Tube 2 and 20 ml. of a saturated solution of NaIO₄ in dilute borate buffer of pH 8.0 are added. A fairly moderate stream of air, controlled with a flowmeter, is used to aerate the acetaldehyde into Tube 3.

A standard solution of threonine is oxidized simultaneously in the companion gas train.

The remainder of the determination is carried out as given in Section A.

Comment: The periodate mixtures of Shinn and Nicolet (578) and of Winnick (685) can be used in place of the one suggested above.

The use of periodate in place of lead tetraacetate is more convenient in almost every respect and is to be recommended except for special purposes.

It should be noted that the p-hydroxydiphenyl method requires approximately 0.03 mg. of threonine, the Conway vessel modification needs 0.3 mg. of threonine, and the titration procedure, 3 mg. of the amino acid per determination.

CHAPTER IV

PART III

THE DETERMINATION OF SERINE

Historical: Although serine was first discovered by direct crystal-lization from a sericin hydrolysate, and later isolated from a num-ber of proteins by the Fischer ester hydrochloride method, the first simple and reliable procedure for the determination of serine in protein hydrolysates is that described by Nicolet and Shinn (476) in 1941.

1. OXIDATION TO FORMALDEHYDE BY SPECIFIC OXIDIZING REAGENTS

A. The Periodate Procedure of Nicolet and Shinn (476)

Principle: The protein hydrolysate is oxidized in neutral or dilute alkaline solution with periodic acid. Acetaldehyde, which is formed from the threonine present, is removed by aeration. The formaldehyde resulting from serine, is retained in solution by the amino groups of other amino acids. After the oxidation is complete and the CH_3CHO has been removed, the HCHO is liberated with acid and precipitated with 5,5-dimethyldihydroresorcinol (Vor-länder, 666).

Method: 1. Hydrolysis. 2 to 5 gm. of protein are hydrolyzed for 24 hours with 20 per cent HCl. The excess acid is removed by re-peated concentration *in vacuo* and the hydrolysate is decolorized with a small amount of activated carbon.

2. Oxidation and Removal of CH_3CHO: An aliquot of the hy-drolysate, containing 10 to 20 mg. of serine, is oxidized in dilute alkaline solution with HIO_4 (*cf.* Part II, Section B, this chapter) using a gas adsorption train of three tubes, one oxidation tube and two tubes containing dilute bisulfite to retain the CH_3CHO.

If a second set of tubes is connected in series, each set should be separated by a gas washing tube containing $NaHCO_3$.

3. Precipitation. After one hour's aeration, the formaldehyde containing solution is transferred to a 300 ml. Erlenmeyer flask and 1–2 drops of methyl red are added. The *p*H of the solution is then carefully adjusted with acetic acid until the color of the solution changes from yellow to a faint red. An excess of 0.4 per cent aque-

ous dimedon (5,5-dimethyldihydroresorcinol) is then added and the precipitate is allowed to form at room temperature for 48 to 72 hours. The precipitate is filtered on a No. 4 sintered glass funnel, washed thoroughly with cold dimedon and dried *in vacuo.*

1 mg. of Methylenedimethyldihydroresorcinol ≈ 0.3596 mg. of Serine.

M.P. 189° (corrected) (Vorländer, 666)

Comment: The modified procedure described by Nicolet (478) for the determination of threonine in the presence of certain sugars is equally applicable to the determination of serine in such mixtures (*cf.* Part II, Section B, *Comment*).

Other substances, such as certain carbohydrates and hydroxylysine, which yield HCHO on periodate oxidation, would of course lead to erroneous values. However, if extraneous formaldehyde is corrected for by Nicolet's acetylation procedure (478), the error due to hydroxylysine is usually small except in the case of collagen and gelatin.

Martin and Synge (438) criticized this procedure as follows, "the dimedon method seems valueless for determining serine, etc. in complete protein hydrolysates . . . ." The authors' experience has not justified this severe criticism.

B. Boyd's Micromodification of the Nicolet-Shinn Method
(119, 120)

Principle: The formaldehyde formed by periodate oxidation of serine is distilled into Eegriwe's 1,8-dihydroxynaphthalene-3,5-disulfonic acid reagent to yield a colored compound.

Reagents: 0.5 M HIO_4:11.4 gm. of $HIO_4 \cdot 2H_2O$ are dissolved in water and the solution is diluted to 100 ml.

Methyl red: a saturated solution in 0.05 N HCl is prepared.

Formaldehyde Standard. 100 gm. of paraldehyde in 100 ml. of H_2O are hydrolyzed with 20 ml. of 2 N H_2SO_4 at 90° until no precipitate remains. The HCHO is then steam distilled and the distillate is returned to the original flask and redistilled after adding 10 ml. of 2 N H_2SO_4. The stock HCHO solution thus obtained should be approximately 6 M. If 1 ml. of 2 N H_2SO_4 per liter is added to the stock solution no perceptible change in the concentration of HCHO takes place over a period of one month.

The HCHO is standardized as follows: An aliquot (0.2 to 0.3 ml.) is added to 20 ml. of 0.12 N KCN. To this 25 ml. of 0.100 N $AgNO_3$ and 3 ml. of HNO_3 are added. The solution is diluted to 100 ml., filtered, and 75 ml. of the filtrate are titrated with 0.1

N NH₄SCN using 5 ml. of saturated $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4$ solution to detect the end point. The KCN solution is standardized by the same procedure except that HCHO is omitted.

The standard is then diluted to contain 15γ per ml. This working standard is prepared fresh daily.

Chromotropic Acid, 1,8-dihydroxynaphthalene-3,6-disulfonic acid, is purified as follows: 25 gm. are dissolved in 100 ml. of hot water. 2 gm. of $PbCO_3$ are added and after solution has occurred, the Pb is removed by H_2S. If the supernatant liquid is not a pale yellow, more $PbCO_3$ should be added. The PbS is removed by centrifugation without contact with air. The chromotropic acid is crystallized out at 4° and the precipitate is washed with alcohol and ether and dried *in vacuo.*

0.1 M Chromotropic Acid: 0.9 gm. are dissolved in 25 ml. of H_2O. 50 mg. of $SnCl_2$ are added and after shaking, the precipitate is removed by centrifuging. The solution is prepared fresh every 48 hours.

Procedure: 1. Oxidation. 4 ml. of 25 per cent $KAsO_2$ and 3 drops of methyl red are introduced into a 300 ml. Kjeldahl flask. The neutralized serine solution (containing 1 to 5 mg. of serine) is now added, followed by 2.5 to 2.8 ml. of 0.5 M HIO_4. The addition of HIO_4 is stopped when the mixture is acid to methyl red. The HIO_4 should be added dropwise with continuous gentle shaking. The solution is diluted to 70 ml. with water.

2. Distillation. The contents of the flask are distilled in 10 to 12 minutes into 5 to 10 ml. of water (the water in the receiver must, of course, cover the end of the distillation tube) until only approximately 5 ml. of fluid remain in the Kjeldahl flask. The iodate, which tends to become dried on the sides of the flask, should be washed down by swirling. The distillate is diluted to 100 ml.

3. Color Development. An amount of distillate, containing 40 to 100γ of HCHO, is placed in a test tube graduated to 50 ml. Then 0.5 ml. of chromotropic acid are added and enough water to make the final volume 17 ml. The tube is cooled in an ice bath and 10 ml. of concentrated H_2SO_4 are added slowly during 40 to 45 seconds. The contents are again cooled to 0° and sulfuric acid is added to the 50 ml. mark. The tube is now heated for 10 minutes in boiling water, cooled to room temperature, and read, within an hour, against a similarly prepared standard or a calibration curve.

Comment: This appealing micromethod is claimed to have an error of only 1 to 2 per cent. Carbohydrates and higher aldehydes do not interfere, but HCHO resulting from the oxidation of hydroxylysine will give erroneously high serine values.

2. Miscellaneous Methods

Fromageot and Heitz (245) have observed that when the hydroxy acids resulting from the deamination of an amino acid mixture are oxidized with $KMnO_4$, lactic acid, malic acid, and glyceric acid yield acetaldehyde, the determination of which permits the approximate estimation of the sum of serine, alanine, and aspartic acid in the hydrolysate.

Rapoport (537) has reported that glycollic acid is formed from serine and glycine by oxidation with diluted $KMnO_4$ following deamination. The sum of the two amino acids is then calculated from the glycollic acid. The method does not appear promising.

PART IV

β-HYDROXY AMINO ACIDS IN PROTEINS

As in the preceding tables all amino acid values given below have been calculated to 16.0 per cent of nitrogen. In contrast to our former practice, however, the method used is designated by the letters F or O. The former indicates that the amino acid, almost without exception serine, was isolated after fractional distillation of the esters according to Fischer. The symbol O refers to one or another of the modifications of the tetraacetate or periodate oxidation methods.

It will be seen that the values obtained by the Fischer method are usually only a small fraction of the quantity estimated by the oxidation procedures. Their purpose serves simply to indicate the presence in the protein of the amino acid in question.

β-Hydroxy Amino Acids in *Animal Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SERINE	THREONINE
				per cent	gm.	gm.
Albuminoids						
Gelatin	O	Block	100	16.0		1.2
Gelatin	O	unpublished				1.5
Gelatin	O	Martin	438	(16.0)		2.0
Gelatin	O	Nicolet	476	(16.0)	3.3	
Gelatin	O	Shinn	578	(16.0)		1.4
Collagen	O	Boyd	120		3.7	
Elastin	O	Brand	128	(17.1)		2.5
Fish Gelatin	O	unpublished		11.8		2.3
Entire Animals:						
Rat	O	unpublished		12.4		4.5
Blood Proteins:						
Fibrin	O	unpublished		13.4		7.9
Globin-Human	O	unpublished		16.2		6.8
Hemoglobin-Horse	O	Boyd	120		5.3	
Hemoglobin-Dog	O	Boyd	120		5.1	
Serum-Human	O	Block	100	14.9		6.4
Serum-Human	O	Block	105	14.9		6.2
Brain Proteins:						
Human	O	Block	105	14.1		5.8
Beef	O	Beach	59		7.1	5.3
Egg Proteins:						
Albumin	O	unpublished		13.9		4.3
Albumin	O	Boyd	120		7.6	
Albumin	O	Martin	438	15.8		3.1
Vitellin	O	Nicolet	479	15.3	9.4	4.9
Whole Egg	O	unpublished		14.1		4.9

β-Hydroxy Amino Acids in *Animal Proteins—Continued*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SERINE	THREONINE
				per cent	gm.	gm.
Feeds						
Meat Scraps	O	unpublished				3.9
Tankage	O	unpublished		10.6		3.5
Menhaden Meal	O	unpublished		11.6		5.1
Skim Milk	O	unpublished				4.7
Hormones, Enzymes:						
Insulin	O	Nicolet	476A	15.7	3.6	2.7
Pepsin	O	Brand	128	15.4		9.9
Trypsin	O	Brand	128	(16.0)		5.8
Chymotrypsinogens	O	Brand	128	(16.0)		10–11
Eukeratins:						
Horn	F	Fischer	224		1	
Hoof	O	unpublished		15.0		4.9
Hair-Hog	O	unpublished		15.1		6.1
Hair-Chimpanzee	O	Winnick	685	16.3		6.6
Pseudokeratins:						
Silk Fibroin	F	Abderhalden	20	19.0	1–2	
Silk Fibroin	O	Martin	438	18.3		0.8
Silk Fibroin	O	Nicolet	475	19.1	11.4	1.3
Silk Serecin	O	Nicolet	475	16.5	32.9	9.8
Liver Proteins:						
Human	O	Block	105	13.6		5.8
Beef	O	Beach	59		7.3	4.8
Cod	O	unpublished				5.4
Milk Proteins:						
Casein:	O	Block	100	14.7		3.8
Casein	O	Borchers	115	(15.4)		3.6
Casein	O	Boyd	120		5.7	
Casein	O	Martin	438	13.4		4.1
Casein	O	Nicolet	476	14.0	5.7	
Casein	O	Nicolet	477	15.8	5.9	4.0
Casein	O	Nicolet	479	15.8	7.5	
Casein	O	Shinn	578	(14.0)		4.0
Casein	O	Toennies	613	14.1	4.8	4.0
Casein Hydrolysate	O	unpublished		12.3	7.6	
Casein Hydrolysate	O	unpublished			6.2	4.3
β-Lactoglobulin	O	Bolling	112	15.5		6.0
β-Lactoglobulin	O	Brand	128	15.6		6.0
β-Lactoglobulin	O	Nicolet	477	15.4		5.4
β-Lactotlobulin	O	Winnick	685	14.4		6.0
Lactalbumin	F	Jones	340	15.4	2	
Lactalbumin	O	Nicolet	476	13.9	4.9	
Lactalbumin	O	Nicolet	477	15.4	4.9	5.4
Lactalbumin	O	unpublished		13.8		5.2
Whole Milk, Cow's	O	unpublished		15.2		4.6
Whole Milk, Human	O	unpublished		15.2		4.6
Muscle Proteins:						
Heart, Beef	O	unpublished		14.8		4.0
Heart, Beef	O	Beach	59		5.9	4.7
Bladder	O	unpublished		16.0		3.3
Intestine	O	unpublished		15.3		3.5
Muscle, Beef	O	unpublished		16.1		3.5
Muscle, Beef	O	Beach	59		5.4	4.6
Muscle, Cod	O	Abderhalden	24	13.6	2	

β-Hydroxy Amino Acids in *Animal Proteins—Continued*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SERINE	THREONINE
			●	per cent	gm.	gm.
Myosin	O	Martin	438	16.8		3.8
Myogen	O	Martin	438			4.2
Veal, Muscle	O	Beach	59		6.1	5.1
Lamb, Muscle	O	Beach	59		6.3	5.3
Chicken, Muscle	O	Beach	59		4.7	4.7
Tissue Proteins:						
Spleen	O	unpublished		15.7		3.8
Thymus	O	unpublished		15.4		4.1
Lung	O	unpublished		15.3		3.8
Lung	O	Beach	59		6.7	3.8
Pancreas	O	unpublished		15.5		4.1
Salivary	O	unpublished		15.7		3.5
Testes	O	unpublished		15.4		3.5
Ovaries	O	unpublished		15.8		3.7
Kidney	O	Beach	59		6.1	4.6
Stomach	O	Beach	59		7.0	3.4

β-Hydroxy Amino Acids in *Plant Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SERINE	THREONINE
●				per cent	gm.	gm.
Biologically Active Proteins:						
Tyrocidine	O	Christensen	162	14.5		0.0
Tobacco Virus	O	Ross	557	15.9	7.8	6.3
Tobacco Virus	O	Ross	556	15.9	6.2	6.3
Corn Proteins:						
Whole, White	O	unpublished				3.7
Whole, Yellow	O	unpublished				3.4
Gluten, White	O	unpublished		10.9		4.1
Gluten, Yellow	O	unpublished		12.7		3.9
Gluten, Meal	O	unpublished				3.2
Germ, White	O	unpublished		11.8		4.7
Germ, Yellow	O	unpublished		12.8		4.0
Zein	O	Borchers	115	15.5		2.5
Zein	O	Martin	438	16.3		2.3
Zein Residue	O	unpublished		10.9		4.0
Bran	O	unpublished				4.0
Albumins	O	unpublished		12.6		3.9
Foods:						
Bread	O	unpublished		11.3		2.8
Flour	O	unpublished		13.1		2.7
"Cerevim"	O	unpublished				2.1
"Wheatena"	O	unpublished		9.5		3.9
"Ralston"	O	unpublished		12.5		3.8
"Cream Farina"	O	unpublished				3.3
"Cream of Wheat"	O	unpublished		13.6		2.7
"Puffed Wheat Sparkies"	O	unpublished				3.8
Leaf Proteins:						
Leaves	O	Martin	438	13.1		4.1
Alfalfa	O	unpublished		10.6		5.4

β-Hydroxy Amino Acids in *Plant Proteins—Continued*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	SERINE	THREONINE
Miscellaneous:				per cent	gm.	gm.
Cottonseed Globulin	O	Fontaine	238	17.2	2.7	2.7
Cottonseed Meal	O	unpublished		10.9		3.0
Linseed Meal	O	unpublished				5.1
Peanut Meal	O	unpublished		10.4		1.5
Peanut-Arachin	O	Brown	133	18.0	4.6	2.3
Peanut-Arachin	O	Martin	438			2.1
Peanut Conarachin	O	Brown	133	18.0	4.4	1.8
Soybean Meal	O	unpublished				4.0
Castor Bean-Ricin	O	Martin	438	(17.0)		2.7
Cocoanut Globulin	F	Johns	334	18.5	1–2	
Yeast-Brewers	O	unpublished				5.0
Yeast-Steep Water	O	unpublished				5.0
Flaxseed Meal	O	unpublished				4.0
Oat Meal	O	unpublished				3.6
Rice Cereal	O	unpublished				3.9
Wheat Proteins:						
Germ	O	unpublished				3.8
Flour	O	unpublished		13.1		2.7
Gluten	O	Martin	438	13.5		2.3
Gliadin	O	Winnick	685	17.6		2.7
Whole Wheat	O	unpublished				3.3

CHAPTER V.

THE "LEUCINES"

LEUCINE, ISOLEUCINE, AND VALINE

	Leucine	Isoleucine	Valine
Empirical Formula	$C_6H_{13}O_2N$	$C_6H_{13}O_2N$	$C_5H_{11}O_2N$
Optical Form	l	d	d
Molecular Weight	131.11	131.11	117.10
Carbon	54.92	54.92	51.24
Hydrogen	9.99	9.99	9.47
Nitrogen	10.69	10.69	11.96
Oxygen	24.41	24.41	27.33
Melting Point	293–5°	280°	315° (cor.)

PART I

INTRODUCTION

IN contrast to the numerous methods given in Chapters I, II, and III for the diamino, aromatic, and sulfur containing amino acids and the relatively simple but accurate oxidation procedures for threonine and serine described in Chapter IV, the procedures for the estimation of leucine, isoleucine, and valine are not only few and difficult to carry out, but the results obtained leave much to be desired.

Although the available evidence, as well as chemical structure, suggest that the "leucines" are not destroyed to any great extent during acid hydrolysis even in the presence of carbohydrates, a definite opinion on this point should not be entertained until further, more nearly quantitative data become available. The experiments of Lyman et al. (433B and private communication) show that the loss of these three amino acids because of humin formation appears to be relatively low.

215

PART II

THE ESTIMATION OF LEUCINE, ISOLEUCINE, AND VALINE

1. The Fischer Ester Hydrochloride Method (222, 224)

Principle: Fischer's ester hydrochloride method is described in greater detail in another place. It suffices to say here that leucine, isoleucine, and valine esters are distilled among the lower boiling fractions (Osborne, Jones and Leavenworth, 497). Leucine and isoleucine are separated from valine by precipitation with lead acetate (Levene and Van Slyke, 415). The quantities of leucine and isoleucine in the mixture are estimated by optical rotation. Valine is then separated from alanine by precipitation of the latter amino acid with phosphotungstic acid (Levene and Van Slyke, 417).

Method (In brief): 1. Hydrolysis. A relatively large quantity of the protein (250 to 1000 gm.) is hydrolyzed with 18 to 36 per cent HCl for 8 to 36 hours. The excess acid is removed by concentration *in vacuo.*

2. Separation of Amino Acids by Crystallization. A fraction of the glutamic acid is removed from the hydrolysate by direct crystallization as the hydrochloride according to Hlaziwetz and Habermann (300). The hydrolysate is neutralized and a portion of the tyrosine crystallizes out.

3. Esterification. The remainder of the hydrolysate is esterified with HCl in absolute alcohol and the mineral acid is removed by one of the following: NaOH–K$_2$CO$_3$, Ba(OH)$_2$, PbO, NH$_3$, or C$_2$H$_5$ONa. The free esters are extracted with ether.

4. Distillation. Osborne, Jones and Leavenworth (497) in a very careful analysis of egg albumin found that valine, leucine, and isoleucine esters were present in the fraction distilling at 100° bath temperature and 10.00 mm pressure. Leucine and isoleucine esters also came over at the next higher fraction, namely 107° bath temperature and 0.40 mm pressure. After hydrolysis of the amino acid esters, the hydrolyzing acid is quantitatively removed and the solution is evaporated to dryness. Proline is extracted from this mixture with ethanol. Leucine, isoleucine, valine, and alanine are present in the residue.

216

5. *Separation of Leucine and Isoleucine from Valine.* Levene and Van Slyke (415) found that leucine and isoleucine could be separated from valine and other amino acids in the low boiling ester distillate by the addition during thorough agitation of a slight excess of the theoretical quantity of lead acetate. They added 4 ml. of 1.1 M lead acetate solution (sp.gr. 1.2540 at 20°) per gm. of leucine plus isoleucine. The amino acids were dissolved in hot water with the least quantity of ammonia. The approximate amounts of the latter amino acids were calculated from a carbon determination where

$$\text{Leucine plus Isoleucine} = \frac{\text{per cent Carbon} - 51.24}{3.68} \times 100.$$

The lead precipitate was removed after cooling for one hour or more and washed with cold 90 per cent ethanol and ether and analyzed for Pb.

6. *Calculation of Leucine and of Isoleucine.* After removal of the lead from the precipitate, the quantities of leucine and isoleucine in the mixture were determined by the optical rotation of the solution in 20 per cent HCl.

$$\text{per cent Isoleucine} = 100 \times \frac{\text{Rotation} - 15.6}{21.8}$$

$$\text{per cent Leucine} = 100 \times \frac{37.4 - \text{Rotation}}{21.8}.$$

7. *Separation of Valine from Alanine.* After removal of the lead from the leucine-isoleucine filtrate, the solution usually contains an inseparable mixture of alanine and valine. Provided that the mixture does not contain more than 50 per cent of valine, Levene and Van Slyke (417) have suggested the following method for their separation.

The amino acids are dissolved in 30 to 40 ml. of 10 per cent H_2SO_4 per gm. of valine. Then the solution is heated to 90° and 14 gm. of phospho-24-tungstic acid (purified according to Winterstein, 686) per gm. of alanine is added. Then 1 gm. more of phosphotungstic acid per 5 ml. of solution is introduced. The solution is cooled at 0° for 24 hours and the precipitate of alanine phosphotungstate is removed.

The precipitate is then dissolved by warming in the same volume of 10 per cent H_2SO_4 from which the original precipitation was carried out. One gm. of phosphotungstic acid per 4 to 5 ml.

of the hot solution is now added and the crystallization is allowed
to take place at 0° for 24 hours. The precipitate is removed and
washed with cold 20 per cent phosphotungstic acid in 10 per cent
H₂SO₄. The precipitate is then decomposed with lead acetate and
after removal of the excess lead with H₂SO₄, alanine is obtained
by crystallization from a small volume.

Levene and Van Slyke report that 90 to 95 per cent of pure al-
anine was recovered under these conditions when a correction of
0.15 gm. of alanine per 100 ml. of phosphotungstic acid solution
was used.

Valine is then obtained from the phosphotungstic acid filtrate
after removal of the inorganic reagents. It is precipitated from
aqueous solution by the addition of acetone to 80 per cent. Only
75 to 85 per cent of valine was recovered by this procedure.

Comment: The great effort involved in obtaining even minimal
values by the Fischer method has resulted in its almost complete
abandonment in recent years. The popularity of this procedure
was, in fact, greatly impaired when Osborne and Jones (501) found,
in 1910, that the method, even in the hands of very adept and ex-
perienced investigators, yielded only 80 to 90 per cent of the leucine
and 40 per cent of the valine present in a mixture of pure amino
acids. Abderhalden and Weil, two years later (19), reported the
recovery of 65 to 75 per cent of leucine and 65 to 70 per cent of
valine under the same conditions.

In spite of all these disadvantages, the method has yielded some
results useful to the nutritionist.

2. The Separation of Leucine from Isoleucine and Valine by Copper Salts (Ehrlich, 207)

Historical: In 1908, Felix Ehrlich and Wendel (207) published
the results of experiments on the separation and isolation of
leucine, isoleucine, and valine from a number of proteins. In the
course of this investigation they observed that the copper salt of
leucine was insoluble in methanol but that one part of isoleucine
copper was dissolved in 55 parts of methanol at 17° and one part
of valine was soluble in 52 parts of CH₃OH at 18°. These results
have been used for the separation of leucine from isoleucine and
valine (Brazier, 129).

A. Brazier's Modification of the Ehrlich Copper Salt Method (129)

Principle: The amino acids are converted into their copper
salts and are thoroughly dried. Isoleucine and valine copper are

extracted with methanol, leucine copper remains in the precipitate.

Method: The protein is hydrolyzed with 8 N H_2SO_4 and the mineral acid is removed with baryta. The amino acid solution is heated to boiling and an excess of copper carbonate is added to the hot solution. The entire solution, including the excess $CuCO_3$, is evaporated to a thick syrup on the steam bath. Then the salts are thoroughly dried with the careful addition of an excess of acetone. The dry salts are pulverized and dried in the oven at 110°C for several hours. The dry salts are ground again if necessary and repeatedly extracted with dry methanol until the filtrate is colorless. The copper salts of isoleucine, valine, hydroxyvaline (?), proline, and some others are present in the filtrate. The greater part of the leucine remains in the precipitate.

Comment: The Ehrlich-Brazier copper method appears promising as a preliminary step for the estimation of leucine, isoleucine, and valine by other methods.

Woolley and Peterson (689) point out that some leucine copper is apt to precipitate along with the excess $CuCO_3$ or $Cu(OH)_2$ used to prepare the copper salts, even in hot dilute aqueous solution. They, therefore, suggest that this fraction should not be discarded as is often done.

Schryver and 'Buston (571) have shown that after heating the dried zinc salts in the oven for some time, leucine zinc becomes insoluble in water, while the valine salt remains soluble.

3. THE SEPARATION OF LEUCINE FROM VALINE AND ISOLEUCINE BY NAPHTHALENE-β-SULFONIC ACID

Historical: Fischer and Bergell (221) found that the naphthalene-β-sulfonyl derivative of *l*-leucine was very insoluble in cold water and soluble to the extent of only one part in 400 of boiling water.

A. The Direct Precipitation of Leucine by Naphthalene-β-Sulfonic Acid (Bergmann and Stein, 71)

Method (as indicated by the paper of Bergmann and Stein): The solution of amino acids in N HCl is heated (80 to 95° ?) and an excess of naphthalene-β-sulfonic acid in an equal volume of hot water is added. The precipitate which is allowed to form at 0° for 3 days, is filtered off and recrystallized from water.

Pure leucine naphthalenesulfonate M.P. 187–189°C

$$N = 3.9 \text{ per cent}$$

Comment: It is claimed that the naphthalenesulfonates of iso-
leucine and valine are much more soluble (71) than that of leu-
cine. This separation, if it can be effected, should, therefore, in-
crease the accuracy of the oxidation methods for leucine and valine
after hydrolysis of the sulfonates.

4. Fractionation of the Acetyl Derivatives of Leucine, Isoleucine, and Valine

A. Counter-Current Liquid-Liquid Separation (Martin and Synge, 437)

Principle: A rather elaborate liquid-liquid extractor is employed
for separating the acetylmonoamino acids by their differential
solubility in chloroform and water. It is claimed that the procedure
leads to a better separation of leucine, isoleucine, and valine than
can be achieved by the distillation of their esters. The reader is
advised to refer to the original paper for the details.

B. The Liquid Chromatographic Method of Martin and Synge (439)*

Principle: A column of silica gel saturated with water is used
as a mechanical support for the aqueous phase in which the ace-
tylated amino acids are separated by the familiar Tswett principle.

Reagents: Silica Gel. One volume of commercial water glass is
dissolved in 2 volumes of water. The SiO_2 is precipitated from solu-
tion by 10 N HCl using methyl orange as the internal indicator.
After standing for several hours, the gel is filtered off and washed
with distilled water until it is free from indicator. The gel is then
aged several days on the filter while wet. It is finally washed again
and dried at 110°.

The silica gel is treated with 70 per cent of its weight (w/w) of
water saturated with methyl orange. The resulting pink powder
should appear dry. 5 gm. of this powder are suspended in 35 ml.
of chloroform previously saturated with water and which contains
1 per cent by volume of normal butanol. The gel, which should now
appear yellow, is poured into a 30×1 cm. tube with a porous plate
at the bottom. A stopper is inserted in the top but the $CHCl_3$
is allowed to run out.

The column should now contain methyl orange firmly held in
the aqueous phase.

Procedure: 1. Hydrolysis. 100 mg. of protein are hydrolyzed with
6 N HCl and the excess acid is removed by concentration *in vacuo.*

* See Chapter IX, Part II, Section 12D.

The hydrolysate is then made alkaline to thymolphthalein with 6 N NaOH and concentrated to a thin syrup.

2. Acetylation. The alkaline solution is treated with 10 ml. of 2 N NaOH and 1 ml. of acetic anhydride in 5 equal portions over 15 minutes. The solution is shaken and cooled in an ice bath between each addition. The solution is allowed to remain alkaline to thymolphthalein for 10 minutes longer and then it is acidified with 1 ml. of 10 N H_2SO_4.

3. Extraction. The acetylated amino acid solution is concentrated *in vacuo* to 5 ml. and then adjusted to acid to thymol blue with 10 N H_2SO_4. It is transferred into a small separatory funnel, final volume 10 ml., and extracted 5 times with 5 volumes of chloroform each time. The chloroform solutions are filtered and the solvent is distilled off. The residue is taken up in 10 ml. of ethanol.

4. Chromatographic Separation. The alcoholic solution, equivalent to 30 mg. of protein, is evaporated to dryness *in vacuo* in a desiccator over H_2SO_4 and soda lime. The residue is dissolved in a minimal quantity of chloroform containing 1 per cent of n-butanol. The $CHCl_3$ solution is carefully transferred to the silica gel column by pipetting down the side of the tube. The bands are developed with fresh solvent. The position of each is revealed by the adsorbed indicator which turns from yellow through orange to pink. As each of the 3 bands passes out of the column, the receiver is changed.

5. Hydrolysis. The chloroform is removed from the fractions and the peptides are split by hydrolyzing under reflux for 3 hours with 2 N HCl.

Comment: The first band is reported to contain phenylalanine; the second, leucine and isoleucine; and the third, valine, proline, and methionine. The method should be useful as a preliminary step in the oxidation procedures to be described below.

Catch, Cook and Heilbron (150) have described an interesting adaptation of this method.

5. THE MICRO OXIDATION METHODS OF FROMAGEOT (246) AND BLOCK (106)

Principle: The hydroxyamino acids, resulting from the deamination of a protein hydrolysate, are oxidized under two different conditions. Both leucine and valine yield acetone but in varying quantities depending upon the conditions of oxidation. This permits the calculation of leucine and valine in a mixture. Isoleucine is estimated from the yield of ethylmethylketone according to Fabinyi's (215) method.

A. The Estimation of Leucine and Valine by Differential Chromate Oxidation According to Fromageot, Heitz, and Mourgue (246, 247)

Principle: The reactions are best illustrated by equations.

$$\begin{array}{c} CH_3 \\ \diagdown \\ CH_3 \diagup \end{array} CHCHNH_2COOH + HONO \rightarrow \begin{array}{c} CH_3 \\ \diagdown \\ CH_3 \diagup \end{array} CHCHOHCOOH$$

$$\begin{array}{c} CH_3 \\ \diagdown \\ CH_3 \diagup \end{array} CHCHOHCOOH + O(H_2Cr_2O_7) \rightarrow CH_3COCH_3 \qquad \text{+other oxidation products}$$

$$\begin{array}{c} CH_3 \\ \diagdown \\ CH_3 \diagup \end{array} CO + 2C_6H_4 \begin{array}{c} CHO \\ \diagdown \\ OH \end{array} + KOH \rightarrow C_6H_4 \begin{array}{c} CH=CH-CO-CH=CH \\ \diagdown \qquad\qquad\qquad \diagup \\ OK \qquad\qquad\qquad KO \end{array} C_6H_4$$

K salt of di-o-hydroxy-dibenzalacetone
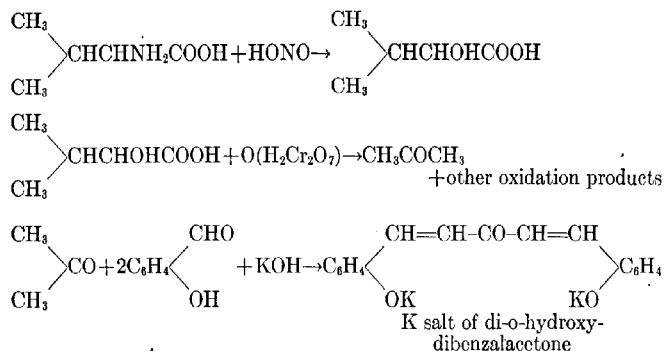
The condensation of acetone with salicylaldehyde in alkaline solution was first proposed by Fabinyi (216) in 1900.

Method: A. *Estimation of Valine in the Absence of Leucine* 1. Deamination. An amino acid mixture containing 2 to 20 mg. of valine and 50 mg. of other amino acids, except leucine, is deaminated in 75 ml. of water with 2.5 ml. of N H_2SO_4 and 15 ml. of 2.5 per cent $NaNO_2$ at 100° for 15 minutes. The excess HONO is destroyed by the addition of 15 ml. of 7.5 per cent urea. The solution is evaporated to less than 10 ml.

2. Oxidation under Pressure. The solution of hydroxy acids is put into a 150 ml. round bottom flask and diluted to 20 ml. with water. Then 4 ml. of glacial acetic acid and 5 ml. of 10 per cent CrO_3 are added. The flask is closed with a ground glass stopper containing a stop-cock. The stopper is wired tightly in place. The flask is placed in boiling water for 2 to 5 hours after which it is cooled under running water and then in an ice bath. The stopcock is opened, and the stopper is replaced with a distilling head. Approximately two thirds of the liquid is distilled into a volumetric flask. The acetic acid in the distillate is neutralized with KOH and the contents are diluted to 100 ml.

3. Direct Oxidation. 5 ml. of 10 per cent CrO_3 are placed in a 150 ml. round bottom flask with a Claisen distilling head attached. The solution of hydroxy acids (volume = 40 to 45 ml.) is allowed to fall drop by drop into the chromic acid which is kept at a gentle

boil. The rate of addition is adjusted so that the volume of the contents in the oxidation flask remains approximately constant during the 25 minute distillation. The distillate, which contains the acetone, is collected in a 150 ml. Erlenmeyer flask which is cooled in ice. 10 ml. of ice water should be present in the flask at the beginning of the distillation. The last traces of the hydroxy acids are rinsed into the oxidation flask with 5 to 10 ml. of water. The distillate is neutralized with KOH and diluted to 100 ml.

4. Determination of Acetone. Urback's modification (624) of Fabinyi's (216) method is used to determine the acetone in the distillate. 2 ml. of 11.3 N KOH (63.6 gm. of KOH in 100 ml. of water) and 1 ml. of alcoholic salicylaldehyde (10 ml. of distilled aldehyde in 142 ml. of 95 per cent ethanol) reagent are added to 2 ml. of the distillate. The solution is placed in a 50° bath for one hour and is mixed from time to time. After 60 minutes, the solution is cooled in an ice bath for 5 minutes and the volume is brought to 15 ml. by the addition of water. The color is read against a standard acetone curve using filter 530 mu. The color was found to be proportional to the acetone concentration over the range 0.08 to 0.80 mg.

5. Yield. Valine gave 61 per cent of the theoretical quantity of acetone under pressure and 69 per cent of the expected amount by direct oxidation. Serine, aspartic acid, glycine, cysteine, isoleucine, glutamic acid, tyrosine and trypophane did not influence the yield of acetone obtained from valine over the range 1 to 12 mg. of valine.

B. *Estimation of Leucine in the Absence of Valine.* This is carried out in the same way as described for valine except that the percentage of expected acetone is different. Thus, Fromageot and Mourgue (247) found that leucine gave 48 per cent of the expected quantity of acetone under pressure and 26 per cent by direct oxidation.

C. *Estimation of Leucine and Valine in Mixtures.* Aliquots of the mixture are deaminated and oxidized by both the "direct" and "pressure" methods. Then if the coefficients of oxidation are known from the results of an adequate number of control experiments on leucine and valine *alone*, determinations of acetone in the distillates will permit the calculation of leucine and valine in the mixture.

Calculations:

If x = the theoretical quantity of acetone from valine
    y = the theoretical quantity of acetone from leucine

a = the quantity of acetone under pressure
b = the quantity of acetone direct
u = the quantity of acetone from valine under pressure
u^1 = the quantity of acetone from valine direct
v = the quantity of acetone from leucine under pressure
v^1 = the quantity of acetone from leucine direct

Then

$$x = \frac{a - vy}{u} \quad \text{and} \quad y = \frac{u^1/u(a - b)}{u^1/u(v - v^1)} \;.$$

If experimentally, $u = 0.611$; $u^1 = 0.690$; $v = 0.479$ and $v^1 = 0.258$, then

$$x = \frac{a - .479y}{.611} \quad \text{and} \quad y = \frac{1.13a - b}{.283}$$

and

$$\text{Valine} = 2.02x; \quad \text{Leucine} = 2.26y.$$

Comment: The values of the four coefficients are empirical depending on the experimental conditions and consequently are subject to variations from one laboratory to another. Fromageot and Heitz (246) stress the point that each investigator *must* determine the 4 oxidation coefficients himself under highly standardized conditions.

Fromageot and Heitz (246) say that it appears possible, then, to obtain with reasonable precision, the quantities of leucine and valine when each comprises 2 to 20 mg. of a mixture of amino acids. The error is about ±26 per cent according to Fromageot and Heitz (246), but only ±5 per cent according to the later paper of Fromageot and Mourgue (247).

B. *The Estimation of Leucine, Isoleucine, and Valine by Differential Oxidation (Block, Bolling and Kondritzer, 106, 104)*

Principle: The amino acid mixture is deaminated with nitrous acid and aliquots of the resulting mixture of hydroxy acids are oxidized with chromate or with permanganate. Leucine and valine are calculated from the yields of acetone after CrO_3 and $KMnO_4$ oxidations essentially according to Fromageot. Isoleucine is calculated from the quantity of ethylmethylketone which is determined by Fabinyi's acid salicylaldehyde reaction (215).

$$2C_6H_4{<}^{OH}_{CHO} +2CH_3\cdot CO\cdot CH_2R \xrightarrow[H_2SO_4]{} 2C_6H_4{<}^{OH}_{CH=CH\cdot CO}{-}^{R\cdot CH_2}{}$$

$$C_6H_4{<}^{OH}_{CH=CH\cdot CO}{-}^{RC\cdot CH_2} + {}^{OC\cdot CH=CH}_{H_2C\cdot R \quad HO}{>}C_6H_4 \xrightarrow[H_2SO_4]{}$$

$$C_6H_4{<}^{OH}_{CH=CH\cdot C}{-}^{R\cdot C=CHC=CH}_{C=CR \quad HO}{>}C_6H_4$$
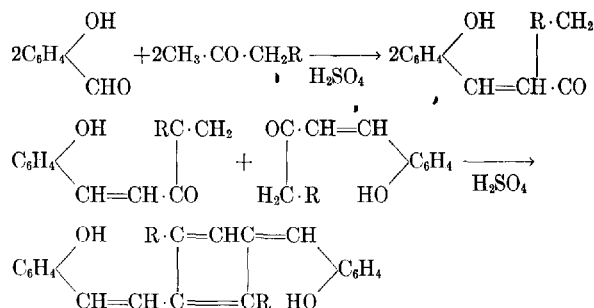
Reagents: 4 N Sodium Nitrite: 28 gm. of $NaNO_2$ are dissolved in 100 ml. of water.

M Phosphate Buffer: 408 gm. of KH_2PO_4 and 685 gm. of $K_2HPO_4\cdot 3\ H_2O$ are dissolved in 6 liters of water. The pH should be approximately 6.8.

Salicylaldehyde Reagent: 16 ml. of redistilled salicylaldehyde (Eastman reagent grade) are diluted to 250 ml. with absolute alcohol. It is advisable to prepare this reagent fresh each day.

15 per cent Mercuric Sulfate: 300 gm. of $HgSO_4$ are suspended in 800 to 900 ml. of 7 N H_2SO_4. 310 ml. of H_2O are added and the suspension is shaken until completely dissolved. The solution is diluted to 2000 ml. with 7 N H_2SO_4 and filtered if necessary. This is 15 per cent $HgSO_4$ in 6 N H_2SO_4 or Denigès' reagent (Folin and Ciocalteu, 232).

Procedure: A. Aeration (Macro)

1. Hydrolysis. Two or 3 gm. of protein are hydrolyzed with 8 to 10 volumes of 18 per cent HCl under reflux for 16 to 20 hours. The hydrolysate is evaporated to dryness to remove the excess acid and any acetone which may have been used to dry the protein. The residue is made up to 50 ml.

2. Deamination. Five or 10 ml. aliquots of the hydrolysate are placed in 125 ml. Erlenmeyer flasks and diluted to 10 ml. with water. The amino acids are deaminated at room temperature with 5 ml. of 4 N $NaNO_2$ and 2 ml. of 1:3 H_2SO_4 for 10 minutes. The excess HONO is then destroyed by warming the flasks on a steam bath for 10 minutes. The solution is neutralized to approximately pH 4 with 20 per cent NaOH

3. Chromate Oxidation. 2 gm. of $K_2Cr_2O_7$ are dissolved in 20 ml. of water in a 3×20 cm. test tube into which a slow stream of washed air is passed in order to aspirate the ketones into 3 water traps connected in series. The water traps are kept in an ice bath

throughout the run. The oxidizing solution is heated to boiling (microburner) and the solution of hydroxy acids (equivalent to not over 50 mg. of protein) is introduced from a burette at such a rate that the volume of solution in the boiling tube remains approximately constant. This addition requires approximately 30 minutes. The aeration and gentle boiling are continued for another 30 minutes until the volume of liquid in the oxidizing tubes has been reduced to less than 10 ml. The aqueous solutions in the traps are combined and diluted to 100 ml.

4. Permanganate Oxidation. 2 gm. of $KMnO_4$ are dissolved in 20 ml. of M phosphate buffer of p_H 6.8. The solution is heated to boiling, a slow stream of air is started through the oxidizing tube and the solution of hydroxy acids (equivalent to not more then 25 mg. of protein nitrogen) is added slowly from a burette. The mode of addition and oxidation is the same as given above in 3. The aqueous solution of the ketones is diluted to 100 ml.

5. Determination of Acetone after Cook and Smith (171). An aliquot of the ketone solution, usually 75 ml. which contains at least 1.5 mg. of acetone, is diluted to 130 ml. with water. Ten ml. of 1:1 H_2SO_4 and 35 ml. of 15 per cent $HgSO_4$ in 6 N H_2SO_4 are added. The solution is boiled vigorously under reflux for 1 hour. The precipitate of acetone mercury sulfate is removed by filtering the hot solution through a tared No. 4 sintered glass crucible. The precipitate is thoroughly washed with water. The crucible is dried at 110° and allowed to cool in a desiccator. The filtrate and washings are set aside for the determination of ethylmethylketone which is not precipitated by $HgSO_4$ in the concentrations employed.

Acetone $= 0.05 \times$ weight of Acetone Mercury Sulfate

6. Determination of Ethylmethylketone. The filtrate, remaining after the precipitation of acetone with $HgSO_4$, is diluted with water so that 1 ml. will contain approximately 0.01 mg. of ethylmethylketone. A suitable aliquot of this solution is diluted with water to 8 ml. and 4 ml. of absolute alcohol are added. The solution is cooled to 0° and 4 ml. of concentrated H_2SO_4 are added. The solution is again cooled in ice water and 2 ml. of alcoholic salicylaldehyde are added. The tube is tightly stoppered, the contents are mixed, and the tube is placed in a water bath at 55° for 18 hours. Any precipitate which may form is removed by centrifugation or is allowed to settle to the bottom of the reading tube. The color is read in an Evelyn or similar photoelectric photometer using a 520 mu filter against a reagent blank prepared in the same way. Due to the inaccuracies of this colorimetric procedure, all estima-

tions for ethylmethylketone should be carried out at least in triplicate and at two levels of solution.

A calibration curve should‣ be prepared with standard ethylmethylketone in dilute $HgSO_4$ over the range 0.005 to 0.05 mg. of ketone.

B. Distillation (Micro.)

1. Hydrolysis. 100 to 200 mg. of protein are hydrolyzed under reflux with 3 to 5 ml. of 1:1 HCl for 16 to 20 hours. The hydrolysate is evaporated to dryness to remove any traces of acetone which may have been left in the protein. The hydrolysate is diluted to 100 ml.

2. Deamination. Ten aliquots of the acid solution, equivalent to 5 to 10 mg. of protein for chromate oxidation and approximately one half this quantity for permanganate oxidation, are pipetted into 125 ml. Erlenmeyer flasks. The volume of each is adjusted to 10 ml. with water. The amino acids are deaminated at room temperature for 10 minutes by the addition of 2 ml. of 4 N $NaNO_2$ and 2 ml. of 1:3 H_2SO_4. The excess HONO is destroyed at the end of the deamination by warming on a steam bath for 10 minutes. The strongly acid solutions are neutralized to approximately pH 4 with 20 per cent NaOH i.e. just acid to Congo red paper.

3. Chromate Oxidation. The solutions of hydroxy acids are then poured into 100 ml. Kjeldahl flasks, the quantitative transfer is effected with the aid of 20 ml. of aqueous 4 per cent $K_2Cr_2O_7$. The hydroxy acids are oxidized by distilling over a low flame using the Hengar microkjeldahl distillation apparatus which is a modification of Folin and Wright's method (229). The distillate is collected in a 300 ml. Erlenmeyer flask which contains 10 ml. of ice water. The receiving flask is cooled in an ice-bath throughout the operation. The distillation is continued until approximately 25 ml. of distillate have been collected in 20 minutes. The distillate is diluted to exactly 50 ml.

4. Permanganate Oxidation. This is carried out with the same apparatus as mentioned above except that 20 ml. of 1 per cent $KMnO_4$ in M phosphate buffer of pH 6.8 is used as the oxidizing agent. In this case, *almost all* of the liquid is distilled over during the course of 45 minutes. The distillate is diluted to 50 ml.

5. Determination of Acetone. An aliquot of the distillate, containing approximately 0.025 mg. of acetone, is pipetted into an Evelyn reading tube ($17 \times 2\frac{1}{2}$ cm. test tube) and diluted to 8 ml. with water. Then 8 ml. of 40 per cent NaOH and 1 ml. of freshly prepared alcoholic salicylaldehyde reagent are added with thorough mixing after each addition. The tube is stoppered tightly,

placed in a water bath at 55° for 30 minutes, and allowed to stand. in the dark at room temperature over night or the color tube is kept at 55° for 2 hours, and read immediately. In both cases, a standard acetone control is run simultaneously. The colors are read against a reagent blank using filter 520 mu.

If the color developed by the standard acetone does not check the calibration curve, the unknown value is corrected accordingly. All estimations should be carried out in duplicate.

The color is proportional to the concentration of acetone over the range 0.005 to 0.04 mg. Ethylmethylketone, except in very great excess, fails to give a color under these conditions (cf. Fromageot and Heitz, 246).

6. Determination of Ethylmethylketone in the Presence of Acetone. A suitable aliquot of the distillate, which contains approximately 0.03 mg. of ketone, is placed into an Evelyn reading tube, diluted to 8 ml. with water and 4 ml. of absolute alcohol are added. The solution is cooled to 0° and 4 ml. of concentrated H_2SO_4 are introduced. The solution is then cooled in ice water and 2 ml. of alcoholic salicylaldehyde are added. The contents are thoroughly mixed after the addition of each reagent. The tube is tightly stoppered and placed in a water bath at 55°C for 18 hours. The color is read against a reagent blank using a 520 mu filter. All estimations are carried out in triplicate and standard ethylmethylketone solutions are analyzed with each unknown to verify the procedure.

Calculations: A. Calculation of Leucine and Valine

When constant conditions of oxidation for the individual amino acids have been established, the following formulae serve to permit the estimation of leucine and valine in a mixture of the two.

$$\text{Acetone }_{K_2Cr_2O_7} = F^1 + F^2$$
$$\text{Acetone }_{KMnO_4} = F^3 + F^4$$

where, Acetone $_{K_2Cr_2O_7}$ is the amount of acetone formed by chromate oxidation and Acetone $_{KMnO_4}$ is the amount of acetone formed during permanganate oxidation and

F^1 is the fraction of valine converted to acetone by $K_2Cr_2O_7$

F^2 is the fraction of leucine converted to acetone by $K_2Cr_2O_7$

F^3 is the fraction of valine converted to acetone by $KMnO_4$

F^4 is the fraction of leucine converted to acetone by $KMnO_4$

Leucine $= 2.26 \times$ Acetone derived from leucine.
Valine $\;= 2.02 \times$ Acetone derived from valine

B. Calculation of Isoleucine from Ethylmethylketone in the Absence of Acetone.

$$\text{Isoleucine} = 1.82 \times \frac{100}{F} \times \text{ethylmethylketone} \quad \text{where } F \text{ is the per}$$

cent of the theoretical yield of ethylmethylketone from isoleucine.

C. Calculation of Isoleucine from a Solution Containing Both Acetone and Ethylmethylketone.

When the microdistillation or microaeration (i.e., the aeration apparatus with one tenth the given quantity of protein and reagents) procedures are employed, the quantities of acetone are too small to permit convenient quantitative precipitation by $HgSO_4$.

When the conditions described above for the estimation of ethylmethylketone by salicylaldehyde in acid solution are used, it has been found that acetone gives only 26 per cent of the color produced by an equal weight of ethylmethylketone. As the latter ketone does not give any color with salicylaldehyde in alkaline solution, it is a simple matter to determine the quantities of both acetone and ethylmethylketone in a mixture of the two.

$$\text{Isoleucine (uncorrected)} = 1.82 \ (E - 0.26 \times \text{Acetone})$$

where E is the total color found by the acid salicylaldehyde reaction read from the ethylmethylketone curve. Acetone can be determined in a separate aliquot of that particular solution by the alkaline salicylaldehyde or any other suitable method.

This calculation gives the uncorrected value for isoleucine. However, as this oxidation, too, is not quantitative, the uncorrected value must be divided by the per cent of ethylmethylketone obtained from known amounts of the amino acid under the established conditions of oxidation.

Comment: Although the oxidation procedure of Fromageot, Heitz, and Mourgue and of Block and Bolling for the microestimation of leucine, valine, and isoleucine leave much to be desired with respect to accuracy and simplicity of operation, they do permit, for the first time, the estimation of these three essential amino acids in small quantities of protein.

In order to reduce the inherently large errors of the method, especially for leucine and valine, it is our present custom to run simultaneously five microdistillations on the unknown and five on a known mixture of leucine and valine. The composition of the latter mixture should approximate that of the unknown. (The determinations on the known mixture are used to confirm the formula.) In this way, 10 to 20 chromate and an equal number of permanganate oxidations are carried out on the unknown. The

yields of ketones are determined and calculated with twice the standard error of the mean (*cf.* Chapter I).

It is obvious that the accuracy of the oxidation procedure is increased with increasing difference in the yields of acetone from leucine by the two methods of oxidation, provided that the yields from valine do not change simultaneously. The aeration procedure yields approximately 50 per cent of the theoretical quantity of acetone from valine and 6 per cent from leucine by chromate oxidation and 50 per cent from valine and 36 per cent from leucine by permanganate oxidation. Isoleucine yields approximately 50 per cent of the expected quantity of ethylmethylketone by both oxidizing agents.

The analogous values for acetone by the microdistillation process are 50 per cent of valine and 9.4 per cent of leucine by chromate, and 64 per cent of valine and 36 per cent of leucine by $KMnO_4$. The yields of ethylmethylketone from isoleucine are 60 per cent and 70 per cent by $K_2Cr_2O_7$ and $KMnO_4$ respectively.

The accuracy of the leucine-valine method would be greatly increased if these two amino acids were separated before oxidation by one of the procedures given in this Chapter.

6. OXIDATION WITH NINHYDRIN

Principle: Leucine, isoleucine, and valine are quantitatively converted into the next lower aldehydes when they are warmed in dilute acid solution with ninhydrin, triketohydrindene hydrate (*cf.* Chapter I, Part IV).

$$\text{(ninhydrin)} + \underset{NH_2}{\underset{|}{RCHCOOH}} \rightarrow RCHO + CO_2 + NH_3 + \text{(reduced ninhydrin)}$$
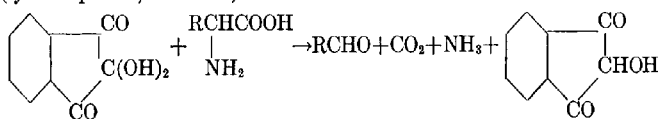
A. Determination of Leucine in Pure Solutions (*Virtanen and Laine, 397 and 662*)

Method: 0.2 to 5.0 mg. of leucine in 10 ml. of solution are gently boiled for 15 minutes with 7.5 gm. of ammonium sulfate, 0.5 gm. of citric acid, and 4 ml. of 1 per cent aqueous ninhydrin. The distillate is trapped in 5 ml. of 1 per cent aqueous $NaHSO_3$. The isovaleraldehyde is quantitatively transferred into the bisulfite by steam distilling for 30 minutes. The bound bisulfite is determined as usual.

Comment: Laine (397) claims that leucine, in the absence of other amino acids which yield volatile aldehydes, can be estimated with an error of less than 5 per cent.

B. The Estimation of the Sum of Leucine, Isoleucine, and Valine
(Virtanen, Laine, and Toivonen, 663)

Principle: When the amino acids of a protein hydrolysate are treated with ninhydrin, valine, leucine, isoleucine, alanine, phenylalanine, and methionine yield volatile aldehydes. There exist separate methods for determining the last three amino acids (*cf.* Chapters II, III, VII); therefore, the sum of the "leucine" group can be calculated.

Method: 1. Hydrolysis and Removal of the Dicarboxylic Amino Acids. Hydrolyze 1 gm. of protein with 10 ml. of concentrated HCl for 6 hours. Remove the excess HCl by evaporation and dilute the residue to 10 ml. Add 5 ml. of milk of lime (the solution must be strongly alkaline), followed by 100 ml. of 96 per cent ethanol with stirring. Discard the precipitate of dicarboxylic amino acids. Remove the alcohol from the filtrate.

2. Estimation of the Sum of Valine, Leucine, and Isoleucine. Dilute an aliquot of the Foreman filtrate (0.05 to 0.5 mg. of N) to 10 ml. with water and add 1 gm. of KH_2PO_4, 1.5 gm. of NaCl, and 2 ml. of 1 per cent ninhydrin solution. Distill the aldehydes into a gas adsorption tube, in an icebath, which contains 3 ml. of 1 per cent $NaHSO_3$. First heat the reaction vessel over a small flame for 15 minutes and then steam distill the aldehydes for 30 minutes longer until a total of 30 ml. has been collected in the receiver.

3. Titration of Total Volatile Aldehydes. Titrate the excess $NaHSO_3$ with 0.1 N I_2 and then exactly remove the excess I_2 with 0.01 N $NaHSO_3$, using starch as the internal indicator. Liberate the bound bisulfite by saturating the solution with $NaHCO_3$. Titrate the free $NaHSO_3$ with 0.01 N I_2.

4. Calculation of the Sum of Leucine, Isoleucine, and Valine. Determine the quantites of aldehyde formed from phenylalanine by the Kapeller-Adler procedure (*cf.* Chapter II), and from methionine by the Baernstein volatile iodide method (*cf.* Chapter III). Calculate the yield of CH_3CHO from alanine by a separate estimation on an aliquot of the Foreman filtrate (*cf.* Chapter VII). The residual aldehyde gives the sum of leucine, isoleucine, and valine.

Comment: This general procedure may be of value when used in conjunction with specific methods of separating leucine, isoleucine, and valine.

Neuberger and Sanger (474) report that it is not necessary to remove the aspartic acid as this substance, contrary to Virtanen, Laine, and Toivonen (663) does not yield some acetaldehyde when warmed with ninhydrin.

C. The Microestimation of Valine (Wretlind, 692)

Principle: Valine is oxidized to isobutylaldehyde by ninhydrin. The aldehyde is steam distilled into water and then determined colorimetrically by Fabrinyi's reaction (216).

Method: 1. Oxidation. 20 to 250 gamma of valine in 10 ml. of water are boiled with 100 mg. of KH_2PO_4, 150 mg. of NaCl and 6 times the calculated quantity of ninhydrin for 5 minutes at 120°. The aldehyde, thus formed is steam distilled for 10 to 15 minutes. The solution is diluted to 10 ml.

2. Development of Color. 2 ml. of exactly 10.5 N NaOH are added to 3 ml. of the aldehyde solution and 1 ml. of 20 per cent by volume of salicylaldehyde is introduced. The mixture is placed in a water bath at 50° for 70 minutes. The tube is then cooled for 10 minutes and the color is read using filter 500 mu.

Comment: It is not stated in Wretlind's paper whether the Fabrinyi reaction is given by the volatile aldehydes arising from leucine, isoleucine, etc. If no color is formed by these with salicylaldehyde, this method should prove most valuable.

7. The Microbiological Determination of Leucine, Isoleucine, and Valine (Lyman, *et al.* 433B; Kuiken, Norman, Lyman, Hale, and Blotter, in press; *cf.* Chapter IX, Part II, Section E.)

Principle: The ability to grow certain microorganisms on synthetic media permits the development of methods for the quantitative determination of each individual essential component of that medium.

Method: Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L.: The Microbiological Determination of Amino Acids. I. Valine, Leucine, and Isoleucine. J. Biol. Chem. 1943, **151**, 615–626.

Basal Medium—The composition of the complete medium for *Lactobacillus arabinosus* (American Type Culture Collection, Georgetown University Medical School, Washington, D.C. No. 8014) is given below. Assay media for valine, for leucine, or for isoleucine are prepared by omitting the appropriate amino acid.

Complete Medium for *Lactobacillus arabinosus*

Glucose	40 gm.	Uracil	10 mg.
Sodium acetate (anhydrous)	14.5 gm.	Thiamine chloride	200 γ
Adenine sulfate	10 mg.	Pyridoxine hydrochloride	200 γ
Guanine hydrochloride	10 mg.	Calcium pantothenate	200 γ

Biotin	0.8 γ	l(−)-Histidine monohydro-	
Riboflavin	400 γ	chloride	400 mg.
Nicotinic acid	800 γ	l(−)-Lysine hydrochloride	400 mg.
p-aminobenzoic acid	1.0 γ	dl-Phenylalanine	400 mg.
Tomato eluate	200 mg.	l(−)-Proline	400 mg.
K₂HPO₄	1 gm.	dl-Serine	400 mg.
KH₂PO₄	1 gm.	l(−)-Trytophane	400 mg.
MgSO₄·7H₂O	400 mg.	dl-Methionine	400 mg.
NaCl	20 mg.	dl-Threonine	400 mg.
FeSO₄·7H₂O	20 mg.	dl-Tyrosine	400 mg.
MnSO₄·4H₂O	20 mg.	dl-Valine	400 mg.
l(−)-Arginine hydrochloride	400 mg.	dl-Leucine	400 mg.
dl-Alanine	400 mg.	dl-Isoleucine	400 mg.
dl-Aspartic acid	800 mg.	l(−)-Cystine	400 mg.
dl-Glutamic acid monohydrate	800 mg.		

Adjust to pH 6.5–6.8 and dilute with water to one liter.

Tyrosine is dissolved in 0.1 N NaOH, cystine in 0.1 N HCl; all other amino acids are dissolved in water and adjusted to neutrality using aqueous phenol red as the internal indicator.

Tomato Juice Eluate—"The contents of a large can of tomato juice (1350 ml.) are diluted with an equal volume of distilled water and centrifuged. The supernatant fluid is clarified by mixing with 120 gm. filter-cel and filtering with suction. The clear filtrate is adjusted to pH 3 with sulfuric acid. Forty grams of Norit A are added and the suspension is shaken mechanically for 30 minutes. The Norit A, on which the active material is adsorbed, is collected by filtering through a thin mat of filter-cel. The Norit is suspended in 250 ml. of 50 per cent ethanol and then collected by filtering through the original mat. The active material is next eluated from the Norit as follows: The Norit and the filter mat are suspended in 200 ml. of a pyridine-ethanol-water mixture (1:2:1 by volume). The suspension is heated to approximately 60° and shaken mechanically for 15 minutes. The eluate is collected by filtering through filter-cel and the Norit is treated with the pyridine-ethanol-water mixture in the same manner two additional times. The filtrates are combined and evaporated to near dryness by vacuum distillation. A small amount of distilled water is added and the solution is neutralized with NaOH to facilitate removal of pyridine. The vacuum distillation is then continued until the pyridine is completely removed.

"Further purification and concentration of the active material is accomplished by hydrolyzing with sulfuric acid in order to break down protein impurities and then repeating the adsorption and elution processes. The material is refluxed for 24 hours with 40 ml. of 8N H₂SO₄. Then a hot saturated solution of barium hydroxide is added until the hydrolysate is basic to congo red but

acid to litmus. The BaSO$_4$ is removed and washed two times with hot water. The hydrolysate and washing are combined and adjusted to pH 3. Any precipitate which forms at this point is removed by centrifuging. The adsorption and elution are then repeated as described above using 8 gm. of Norit A and 100 ml. portions of the elution mixture. After removal of the pyridine-ethanol-water mixture by vacuum distillation, a water solution containing 5 mg. of dry solids per ml. is prepared. During the elution some of the Norit may become colloidal and hence extremely difficult to filter. This difficulty can be overcome by adding a little fresh Norit."

Assay Procedure—Stock solutions of *Lactobacillus* are carried as stabs on 0.8 per cent agar, 1 per cent peptonized milk, 1 per cent tryptone, and 200 ml. of filtered tomato juice per liter. The stabs are transferred every month and incubated for 24 hours at 35° and the new cultures are stored in the refrigerator. Cultures for assay are grown on the above medium without agar and after 24 hours incubation, the cells are centrifuged asceptically, washed once with 0.9 per cent saline and then suspended in saline. One drop of a very dilute saline suspension is used for each assay tube.

Graded quantities of the amino acid solution are added in duplicate to a series of tubes. The range of standards is from 0.0 to 0.1 mg. of *dl*-valine, *dl*-leucine, and *dl*-isoleucine. Successive tubes should not differ by more than 0.02 mg. At least 3 levels of the neutralized (pH 6.5–6.8) protein hydrolysate are used. Five ml. of the appropriate assay medium are then added to all tubes and then water to bring the final volume to 10 ml. The contents are mixed, plugged with cotton and sterilized in an autoclave for 15 minutes. The tubes are cooled, inoculated, and incubated at 35° for 72 hours.

The contents are then centrifuged, and 5 ml. aliquots are titrated with 0.1 N NaOH using bromthymol blue as the internal indicator. The quantities of lactic acid formed in the unknowns and in the standard solutions are used to estimate the amount of the amino acid present in the unknown.

Comment: The protein must be completely hydrolyzed. However, if during the hydrolysis a portion of the amino acid is racemized, the analytical results will be low, as *Lactobacillus arabinosus* is only able to utilize the natural form of the essential amino acid.

PART III

LEUCINE, ISOLEUCINE, AND VALINE IN VARIOUS PROTEINS ,

As in the preceding chapters, all values given in the following tables have been calculated as gm. of amino acid per 16.0 gm. of nitrogen. In many instances, especially when the Fischer ester distillation was employed, the values for leucine reported in the literature include isoleucine. These cases have been indicated in the tables (*cf*. Abderhalden and Weil, 18).

In spite of the obvious difficulties in the Fromageot oxidation procedure for leucine and valine, the agreement among various independent investigators using this and the more accurate isotope dilution method is good (*cf*. the analyses of hemoglobin). The oxidation method, which permits an estimation of the quantities of leucine, isoleucine, and valine in as little as 100 mg. of protein, appears to the authors to be somewhat more accurate and many times easier than the only other well described procedure, namely that of Fischer. The microbiological procedure of Lyman *et al.* (433B) and the chromatographic methods of Gordon, Martin, and Synge (261 etc.) are recommended.

The Fromageot values reported in the tables followed by the ± sign have been calculated from 6 or more determinations where the range is indicated by twice the standard error, P = 0.05 (*cf*. Chapter I). These are considered to be more accurate than those values given without this range.

Leucine, Isoleucine and Valine in *Animal Proteins*

Calculated to 16 0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	LEUCINE	ISO-LEUCINE	VALINE	
Albuminoids:				per cent	gm.	gm.	gm.	
Gelatin	Fromageot-Block	unpublished		16.6	3.7±0.5	1.1±0.2	2.1±1.0	macro
Gelatin	Dakin	Dakin	185	18.0	6.8.	0.0	0.0	
Gelatin	Fischer	Fischer	222	(16.0)	2.1		1	a
Gelatin	Fischer	Kossel	383	(16.0)			+	
Gelatin	Bergmann	Stein	588A	(18.3)	3.0			
Gelatin	Chromatographic	Gordon	261B		5.9		2.3	a
Fish Gelatin	Fromageot-Block	unpublished		11.8	2.3±1.9	1.1±0.1	3.4±0.5	
Elastin	Ehrlich-Brazier	Stein	586	17.1	23.1		12.6	a
Collagen	Bergmann	Stein	588A	(18.6)	3.6			
Gelatin	Lyman	Lyman	433B	(16.0)	3.3	1.7	2.5	
Gelatin	Lyman	Beveridge	706	18.2	3.3	2.6	2.0	
Gelatin	Lyman	unpublished			3.1	1.7	2.8	
Gelatin	Neurospore	Ryan	762	16.0	3.6			
Entire Animals:								
Rat	Isotope	Schoenheimer	567		10.8			

Leucine, Isoleucine and Valine in *Animal Proteins* (Continued)

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	LEUCINE	ISO-LEUCINE	VALINE	
				per cent	gm.	gm.	gm.	
Blood:								
Hemoglobin-Horse	Fromageot-Block	unpublished		16.7	16.8±3.0	1.4±0.2	6.5±0.7	macro
Hemoglobin-Horse	Fromageot-Block	unpublished		16.7	16.6±2.4	1.6±0.3	9.1±1.1	
Hemoglobin-Horse	isotope	Foster	705	16.8	14.4			
Hemoglobin-various	Fromageot	Roche	552	(16.7)	16.4±1.6		8.9±1.2	
Hemoglobin	isotope	Ussing	626	(16.7)	16.3			
Globin-Human	Fromageot	Fromageot	247	14.9	17.2		9.8	
Globin-Beef	Lyman	unpublished			15.5	<0.1	7.7	
Globin-Human	Lyman	unpublished				0.1	8.9	
Globin-Human	Lyman	Devlin	704		18	0.3	12.8	
Globin-Rabbit	Lyman	unpublished				1.7	8.8	
Hemoglobin-Pig	Lyman	unpublished				1.6	8.7	
Hemoglobin-Sheep	Lyman	unpublished				0.3	9.2	
Hemoglobin-Turtle	Lyman	unpublished				4.1	7.1	
Hemoglobin-Horse	Lyman	McMahan	708	(16.7)			8.4	
Fibrin-Cattle	Fromageot-Block	unpublished		13.4	14.3±3.9	5.0±0.5	3.9±1.8	macro
Serum Proteins	Fromageot-Block	unpublished		15.0	18	2.7±0.2	6	
Bence-Jones	Fischer	Abderhalden	11	(16.0)	11			a
Bence-Jones	Fischer	Hopkins	308	16.2	5.4	1	5.5	
γ-Globulin	Lyman	unpublished		14.2	8.2	3.3	10.1	
Brain:								
Animal	Fromageot-Block	unpublished		14.2	13.4±2.2	3.6±0.3	4.9±0.7	
Beef	Lyman	Schweigert	711		7.4	5.1	4.8	
Egg Proteins:								
Whole	Fromageot-Block	unpublished		14.0	19.0±2.1	5.3±0.3	4.4±0.6	
Albumin	Fischer	Osborne	497	15.5	11.1		<3	a
Albumin	Ninhydrin	Virtanen	663	12.1		→24←		ab
Albumin	Bergmann	Stein	588A	(15.5)	9.4			
Vitellin	Fischer	Abderhalden	13	(16.0)	11		<3	a
Vitellin	Fischer	Osborne	495	16.3	10		2	a
Livitin	Ehrlich-Brazier	Jukes	348	15.5	11		10	b
Egg Shell Membrane	Fischer	Abderhalden	14	(16.0)	<8		>1	a
Albumin	Lyman	McMahan	449A				6.8	
Whole	Lyman	unpublished		11.2	9.2	8.0	7.3	
Feeds and Foods:								
Menhaden Meal	Fromageot-Block	unpublished		11.6	10	4.0±2.2	4	
Tankage	Fromageot-Block	unpublished		10.6	13	2.5±0.2	6	
Skim Milk	Fromageot-Block	unpublished			13	5	5	
Shark Meat	Lyman	unpublished			6.4	5.2	4.3	
Fish Meal	Lyman	unpublished		11.4	7.1	6.0	5.8	
Keratins:								
Wool	Fischer	Abderhalden	16	(16.6)	11		<3	a
Wool	Martin	Martin	437		10.8		4.6	a
Wool	Chromatographic	Martin	439		11.1			
Wool	Chromatographic	Gordon	261B		9.3		5.2	a
Hair-Horse	Fischer	Abderhalden	9	(16.0)	7.1		1	a
Hair-Rabbit	Fromageot-Block	unpublished			7.1±2.0		2.7±0.7	
Hair-Hog	Fromageot-Block	unpublished		15.1	9.5±1.0	3.6	6.3±0.6	
Horn-Cattle	Fischer	Abderhalden	16	(15.1)	>16		<5	a
Horn-Cattle	Fischer	Fischer	224	(15.1)	19.2		6	a
Hoof-Cattle	Fromageot-Block	unpublished		14.8	15.4±1.6	4.4	5.0±0.5	
Feathers-Goose	Fischer	Abderhalden	10	(16.0)	8		<1	a
Spongin	Fischer	Abderhalden	12	(16.0)	<8			a
Silk Fibroin	Fischer	Abderhalden	20	19.0	2.1			
Silk Fibroin	Bergmann	Stein	588A	(19.0)	0.8			
Silk Fibroin	Lyman	McMahan	449A	(19.0)			2.7	
Scyllium stellare	Fischer	Pregl	529	15.1	6.1			a

Leucine, Isoleucine and Valine in *Animal Proteins* (Continued)

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	LEUCINE	ISO-LEUCINE	VALINE	
				per cen t	gm.	gm.	gm.	
Metallo Proteins:								
Muscle Globins	Fromageot	Roche	552	(16.0)	15		4 to 5	
Hemocyanins	Fromageot	Roche	552	(16.0)	10.2±2.4		5.8±1.3	
Milk Proteins:								
Casein	Fischer	Foreman	240	15.6	10.0		8.1	a
Casein	Fromageot	Fromageot	247	15.0	12.1		7.0	
Casein	Fischer	Levene	415	(15.4)	9	<2	7	
Casein	Fischer	Levene	416	(15.4)	7.2	1.5	7.0	
Casein	Fischer	Osborne	502	15.5			7.5	
Casein	Dakin	Plimmer	523	14.1	13.6			a
Casein	Ninhydrin	Virtanen	663	13.5	←————22→			ab
Casein	Lyman	Lyman	433B	(15.0)	9.9	6.5	6.7	
Casein	Lyman	Hegsted	728	(15.0)	7.9		5.4	
Casein	Lyman	McMahan	708	(14.5)			7.4	
Casein	neurospora	Regnery	709	(14.5)	12–14			
Casein	neurospora	Ryan	708	(14.5)	10.8			
Casein	Lyman	Schweigert	711	(14.5)	10.6			
Lactalbumin	Fischer	Jones	340	15.4	15		<4	a
Lactalbumin	Lyman	unpublished			10.4	6.4	6.4	
β-Lactoglobulin	Fromageot-Block	unpublished		15.4	17.7±4.2	6.6±0.6	7.9±1.4	
β-Lactoglobulin	isotope	Foster	721A	15.6	16.1		6.2	
β-Lactoglobulin	Lyman	McMahan	751	(15.0)			6.2	
β-Lactoglobulin	neurospora	Ryan		(15.5)	15.9			
Milk-Cow	Fromageot-Block	unpublished		15.2	12.2±3.1	4.5±0.4	4.5±0.4	
Milk-Cow	Fromageot	Williamson	712	16.0	16.8	5.4	5.4	
Milk-Human	Fromageot-Block	unpublished		15.2	12.1±3.9	5.2±0.3	5.5±1.5	
Milk-Human	Fromageot	Williamson	712	16.0	16.2	5.3	4.7	
Miscellaneous:								
Lens	Fischer	Hijikata	298	(16.0)	7		1	a
Protamine	Ehrlich-Brazier	Hirokata	299				6.5	
Thymus Histone	Lyman	unpublished			6.9	5.6	4.2	
Insulin	neurospora	Ryan	710	(15.5)	14±1			
Muscle:								
Cod	Fischer	Abderhalden	24	13.6	9	2	<5	
Fish	Fischer	Osborne	493	(16.0)	10.3		1	a
Scallop	Fischer	Osborne	496	17.1	8		4–6	a
Animal	Fromageot-Block	unpublished		15.4	12.1±1.1	3.4±0.2	4–6	
Ox	Fischer	Osborne	498	11.7			<1	a
Rabbit Myosin	Fischer	Sharp	575		10.6		3	a
Horse-Meat	Lyman	unpublished			8.0	6.3	5.8	
Veal	Lyman	Schweigert	711		6–9	5–6	5–6	
Lamb	Lyman	Schweigert	711		8	6	5	
Pork	Lyman	Schweigert	711		7–8	5–6	5–6	
Beef	Lyman	Schweigert	711		8	6	5	
Tissue:								
Glands	Fromageot-Block	unpublished		15.1	11.8±2.0	3.4±0.2	4.0±0.6	
Beef-Liver	Lyman	Schweigert	711		8.4	5.6	6.2	
Beef-Heart	Lyman	Schweigert	711		8.4	5.2	6.3	
Beef-Kidney	Lyman	Schweigert	711		8.0	5.6	5.3	
Beef-Tongue	Lyman	Schweigert	711		7.7	5.7	5.0	
Thymus Histone	Lyman	unpublished			6.9	5.6	4.2	

a Leucine and Isoleucine

b Isoleucine and Valine

ab Leucine, Isoleucine, and Valine.

Leucine, Isoleucine, and Valine in *Plant Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	LEUCINE	ISO-LEUCINE	VALINE	
				per cent	gm.	gm.	gm.	
Corn Proteins:								
Whole Corn	Fromageot-Block	unpublished		12.7	21.5±2.4	3.6±0.3	4.6±0.7	
Germ	Fromageot-Block	unpublished		11.8	16.3±3.1	3.7±0.4	5.8±1.2	
Germ	neurospora	Hodson	707		6.7			
Gluten	Fromageot-Block	unpublished		12.7	24.7±3.7	4.9±0.3	4.6±1.4	
Bran	Fromageot-Block	unpublished			10.3±3.4	3.7±0.4	4.8±2.3	
Zein Residue	Fromageot-Block	unpublished		10.9	11.0±2.9	2.0±0.3	5.5±1.0	
Zein	Fromageot-Block	unpublished		15.3	22.0±1.4	4.2±0.4	1.7±0.5	
Zein	Fromageot-Block	unpublished		15.3	25.5±2.5	4.3±0.3	2.8±0.9	
Zein	Fromageot-Block	unpublished		15.3	23.6±2.4		2.7±1.3	
Zein	Ehrlich-Brazier	Brazier	129	17.5	24.6		9.2	a
Zein	Dakin	Dakin	189	16.1	26	0	0	b
Zein	Dakin	Dakin	189	16.0			1	
Zein	Fischer	Osborne	490	16.1	18.2		<1	a
Zein	Fischer	Osborne	499	16.1	19.6		1.9	a
Zein	Fischer	Osborne	500	16.1	18.8			a
Zein	Ninhydrin	Virtanen	663	14.5		‹26›		ab
Albumins	Fromageot-Block	unpublished		11.5	11.3±4.1	1.3±0.4	2.5±1.1	
Gluten Meal	Fromageot-Block	unpublished			19.6±3.8	3.7±0.3	1.5±0.7	
		c						
Leaf Proteins:								
Alfalfa	Fromageot-Block	unpublished		10.6	11	5.4±0.2	6	
Miscellaneous:								
Bread	Fromageot-Block	unpublished		11.3	11.2±1.6	2.8±0.3	3.1±0.5	
Cottonseed Globulin	Fromageot-Block	Fontaine	238	16.7	8.1	2.f	5.8	
Cottonseed Globulin	Fromageot-Block	Fontaine	238	16.9	8.5	2.3	5.8	
Cottonseed Globulin	Fromageot-Block	Fontaine	238	17.9	7.5	2.2	6.7	
Cottonseed Flour	Lyman	Lyman	433B	6.8	5.0	3.4	3.7	
Coconut Globulin	Fischer	Jones	339	18.5	6		4	a
Edestin	Fromageot	Fromageot	247	18.0	6.6		5.1	
Edestin	Fischer	Levene	416	(18.6)	7		5	a
Edestin	Lyman	Hegsted	768	(18.4)	4.7		4.1	
Flaxseed Meal	Fromageot-Block	unpublished			<5	1.5±0.2	7.5±0.6	
Gramicidin	Bergmann	Hotchkiss	310	14.8	24.0		22.2	
Gramicidin	Chromatographie	Gordon	261Y		30.3		22.2	
Hordein	Fischer	Kleinschmitt	369	17.2	7		<2	a
Kafirin	Fischer	Jones	339	16.4	15		4	a
Legumelin	Fischer	Osborne	492	18.0	9		1	a
Lupin Meal		Heinrich	286	?	9		<2	a
Linseed Meal	Fromageot-Block	unpublished			7.5±2.8	3.4±0.3	5.8±1.3	
Mold	Ehrlich-Brazier	Woolley	689	5.15	4.3	0.7	0.2	
Ricin	Fischer	Karrer	355	(17.0)	15		2	a
Soybean Meal	Fromageot-Block	unpublished			7.7±0.8	4.0±0.4	4.5±0.4	
Soybean Meal		Heinrich	286	?	9		<2	a
Soybean Meal	Lyman	Lyman	433B	7.0	6.6	4.7	4.2	
Virus	Ehrlich-Bergmann	Ross	557	15.9	7.1		4.6	
Tyrocidine	Chromatographie	Gordon	261E		11–13		8.7–10.7	
Tyrocidine	Lyman	Christensen	713		12.3		10.2	
Sunflower Seed	Lyman	Block	701		6.2	5.2	5.2	
Sesame Seed	Lyman	Block	701		7.5	4.8	5.1	
Rice, White	Lyman	unpublished			9.0	5.3	6.3	
Peanut Proteins:								
Meal	Fromageot-Block	unpublished		10.4	7.6±2.4	2.7±0.3	7.5±0.7	
Meal	Lyman	Lyman	433B	7.2	5.5	3.4	4.0	
Arachin	Fischer	Johns	322	18.3	<4		1	a

Leucine, Isoleucine and Valine in *Plant Proteins* (Continued)

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITRO-GEN	LEUCINE	ISO-LEUCINE	VALINE	
				per cent	gm.	gm.	gm.	
Wheat Proteins:								
Whole Grain	Lyman	Lyman	433B	2.3	5.8	3.3	3.6	
Germ	Fromageot-Block	unpublished			7.4±2.3	3.0±0.5	4.1±1.0	
Flour	Fromageot-Block	unpublished		12.6	12.0±2.6	3.7±0.2	3.4±0.5	
Wheat Flakes	Lyman	unpublished			9.1	4.5	5.0	
Gliadin	Fischer	Osborne	489	17.7	5		<1	a
Gliadin	Fischer	Osborne	503	17.7	6		3	a
Gliadin	neurospora	Ryan	710	(17.1)	6.1			
Glutenin	Fischer	Osborne	489	17.5	6		<1	a
Gluten	Padoa		508	?	9		>3	a
Breakfast Foods	Fromageot-Block	unpublished			10	4.2±0.2	5.0±0.8	
Yeast Proteins:								
Mixed	Fromageot-Block	unpublished			13.2±2.6	3.4±0.2	4.4±0.8	
Brewer's	Microbiological	unpublished			7.3	6.0	5.3	
Yeast-Maximum	Lyman	Block	702		8.5	6.2	5.9	
Yeast-Minimum	Lyman	Block	702		6.1	5.5	4.6	
Yeast-Brewer's	neurospora	Hodson	707		6.1	●		

a Leucine and Isoleucine.
b Isoleucine and Valine.
ab Leucine, Isoleucine, and Valine.

THE DICARBOXYLIC AMINO ACIDS

ASPARTIC ACID AND GLUTAMIC ACID

	Aspartic Acid	Glutamic Acid
Empirical Formula	$C_4H_7O_4N$	$C_5H_9O_4N$
Melting Point	251°	197–198°
Molecular Weight	133.07	147.08
Carbon	36.08	40.80
Hydrogen	5.31	6.17
Nitrogen	10.53	9.50
Oxygen	48.11	43.51
Optical Form	l	d

PART I

HYDROLYSIS: The authors are not familiar with any experiments on the losses of glutamic and aspartic acids during hydrolysis. Woodward, Reinhart, and Dohan (687) have shown that only 50 to 70 per cent of the glutamic acid present in a protein hydrolysate could be recovered following the removal of humin by treatment with Cu_2Cl_2 or after extraction of the mono-aminomonocarboxylic acids (sic) with butyl alcohol. Calvery (139) has shown that butyl alcohol extracts a portion of the dicarboxylic amino acids as well as the monoaminomonocarboxylic acids.

Is is expected that since the recent introduction of rapid micro procedures for the quantitative determination of glutamic acid in protein hydrolysates by Cohen (167) and others, the question of hydrolytic losses of this amino acid will be investigated.

β-Hydroxy Glutamic Acid: The large quantities of β-hydroxy-glutamic acid which have been reported (184) to occur in casein and other milk proteins could not be found by Nicolet and Shinn (477) using their highly specific periodate method. However, evidence that a small quantity of this amino acid may exist in casein (0.33 per cent) has been presented by Gulland and Morris (271). Bailey, Chibnall, Rees, and Williams (158) have presented evidence that even this small quantity may have been erroneous.

1. The Isolation of Aspartic and Glutamic Acids

A. Direct Isolation of Glutamic Acid (Hlaziwetz and Habermann 300)

Historical: In 1873, Hlaziwetz and Habermann (300) showed that glutamic acid hydrochloride could be isolated directly by concentrating a casein hydrolysate which had been prepared by boiling with 18 to 20 per cent HCl containing approximately 19 per cent of $SnCl_2$.

Comment: Although, this general procedure has been used for many years, Osborne and Jones (501) pointed out, as long ago as 1910, that the separation of glutamic acid as the hydrochloride depended on conditions not clearly understood and that check results may not be significant but only fortuitous.

B. Ester Distillation (Fischer, 220)

Principle: Glutamic and aspartic acids have been isolated from the high boiling ester fractions by the Fischer method (220) and from the undistilled residue. This method will not be described as it seems to have been completely abandoned since Osborne and Jones (501) showed, by recovery experiments with the pure amino acids, that they could account for only 40 to 50 per cent of the original aspartic acid. Abderhalden and Weil (18, 19), somewhat later, reported recoveries of glutamic acid of 75 per cent or less and of aspartic acid of 45 to 55 per cent by the Fischer ester method.

Abderhalden and Weil (18) in 1911, said that to estimate best the amino acid values, recovery experiments with the pure compounds must be run. They also pointed out that an amino acid method which works well for one protein may not necessarily be the best procedure to use in the analysis of another. This advice holds good today, but is often not followed.

2. Precipitation of Calcium Aspartate and Calcium Glutamate by Aqueous Alcohol (Ritthausen, cf. 649 and Foreman, 239)

Historical: In 1869, Ritthausen (cf., 649) applied Scheele's 1793 method for precipitating organic acids as their calcium salts with alcohol, to the isolation of glutamic and aspartic acids. Ritthausen's

procedure was forgotten and independently rediscovered by Foreman (239) in 1914. The calcium salt-alcohol method is commonly called Foreman's method.

A. Foreman's Modification of the Ritthausen Procedure (239)

Method: 1. Hydrolysis. 20 to 40 gm. of protein are hydrolyzed for 48 hours with 3 volumes of concentrated HCl. The excess acid is removed by evaporation *in vacuo.* The residue is taken up in 200 to 400 ml. of water.

2. Precipitation of Calcium Salts. A good excess of $Ca(OH)_2$, as a cream, is added to the hydrolysate. At least 500 mg. of CaO should be used per gm. of protein. The excess lime is removed by filtration and the precipitate is thoroughly washed with water. The filtrate and washings are concentrated *in vacuo* until 3.5 to 4.0 ml. of the solution contains the equivalent of 1 gm. of protein. Then 96–97 per cent ethanol is added slowly with stirring to complete precipitation. The precipitate of calcium salts is filtered off and washed with ethanol.

3. Decomposition of Calcium Salts. The precipitate is dissolved in 200 to 400 ml. of water and the calcium ion is removed with a very slight excess of oxalic acid. Any chloride is removed with Ag_2SO_4. After removal of the reagents, an excess of aqueous phospho-24-tungstic acid is added. Any precipitate is removed and the phosphotungstic acid is removed by the addition of an excess of warm barium hydroxide. The Ba^{++} is precipitated with the exact quantity of H_2SO_4.

4. Extraction of Pyrrolidone Carboxylic Acid. The amino acid solution is evaporated to dryness and the residue is extracted with glacial acetic acid, to dissolve pyrrolidone carboxylic acid. This can also be measured by the difference between total N and amino N.

5. Calculation of Aspartic and Glutamic Acids. The quantities of aspartic and glutamic acids present in the residue can, after removal of the acetic acid, be calculated as follows:

$$\frac{\%C - 40.80}{\%C - 36.08} = \frac{1}{R} \qquad \text{Aspartic acid} = \frac{1}{R} \times \text{weight of mixture.}$$

$$\text{Glutamic acid} = \frac{R - 1}{R} \times \text{weight of mixture.}$$

6. Isolation of Copper Aspartate. The residue from 4 is dissolved in hot water and an excess of $CuCO_3$ is added to the boiling solution. Copper aspartate crystallizes out.

7. Isolation of Glutamic Acid Hydrochloride. Copper is removed

from the copper aspartate filtrate with H_2S. Then HCl is added and the solution is evaporated to a small volume. Glutamic acid hydrochloride crystallizes out.

Glutamic Acid $= 0.80 \times$ glutamic acid hydrochloride.
Aspartic Acid $= 0.673 \times$ copper aspartate.

Comment: If only a small quantity of aspartic acid is present, it is easier to first remove the glutamic acid as the hydrochloride and then precipitate the aspartic acid as the copper salt.

Foreman (239) pointed out that the degree of precipitation of the dicarboxylic acids depends on their concentration with respect to water in the initial solution. Thus 1 gm. of glutamic acid in 20 ml. of water, resulted in a recovery of 99 per cent. One gm. of glutamic acid in 30 ml. of water gave a recovery of only 83 per cent.

The glutamic acid figures must be corrected for pyrrolidone carboxylic acid formation.

B. Jones and Moeller's Modification of the Ritthausen-Foreman Method (343)

Principle: Barium hydroxide, as suggested by Dakin (185), rather than calcium hydroxide is used to form the amino acid salts.

Procedure: 1. Hydrolyze 50 gm. of protein with 200 ml. of HCl for 30 to 36 hours.

2. Remove the HCl by concentration *in vacuo.*

3. Filter to remove the humin.

4. Precipitate the bases with phospho-24-tungstic acid according to Van Slyke (*cf.* Chapter I).

5. Remove the phosphotungstic acid from the filtrate with amyl alcohol and ether (*cf.* Chapter I).

6. Concentrate the amino acid solution to a syrup and take up in 300 ml. of water.

7. Add an excess of baryta until the solution is alkaline to litmus. Remove and wash the precipitate.

8. Saturate the solution with $Ba(OH)_2$.

9. Pour the alkaline solution (600 ml. volume) into 5 volumes of ethanol with stirring. Stand 48 hours.

10. Wash the precipitate with ethanol.

11. Dissolve the precipitate in 350 ml. of water and precipitate the barium salts with 400 ml. of 95 per cent ethanol.

12. Remove and wash the precipitate. Dissolve it in water and remove the Ba^{++} with H_2SO_4.

13. Concentrate the solution to a small volume and saturate it with HCl gas at $0°$. Glutamic acid hydrochloride crystallizes out.

14. Remove the HCl from the filtrate with Ag_2SO_4.

15. Remove the reagents and boil the aspartic acid solution with $CuCO_3$. Cool several days. Filter off the copper aspartate.

16. Concentrate the filtrate to a small volume to obtain a second crop of copper aspartate.

17. Remove the copper from the filtrate and isolate a second crop of glutamic acid hydrochloride.

Comment: As copper glutamate is somewhat insoluble in water, care must be taken that this salt does not contaminate the second crop of copper aspartate.

Jukes (348) evaporated the solution of copper salts to dryness and then extracted any copper glutamate with 6 one hundred ml. portions of water at room temperature.

Miller (452) avoided the use of silver sulfate to remove HCl from the glutamic acid hydrochloride filtrate by the simple expedient of precipitating the dicarboxylic acids with barium hydroxide and alcohol as in the initial step.

The removal of HCl from the filtrate of the glutamic acid hydrochloride precipitate is, however, unnecessary.

*C. Chibnall's Modification of the Ritthausen-Foreman Method (157)

Procedure: 1. Hydrolysis and Removal of Ammonia. Boil 50 gm. of protein with 300 ml. of 1:1 HCl for 20 to 24 hours. Remove as much of the HCl as possible by concentration *in vacuo*, dissolve the residue in water and add an excess of cream of lime. Filter and wash the precipitate. Concentrate the alkaline solution *in vacuo* to remove ammonia.

2. Precipitation of the Calcium Salts. Concentrate the filtrate to 400 ml., the solution should be strongly alkaline to phenolphthalein at this point, and add 3420 ml. of absolute ethanol with stirring. Filter the resulting precipitate immediately. Wash with absolute ethanol.

3. Reprecipitation of Calcium Salts. Dissolve the washed precipitate in 300 ml. of water and add 10 ml. of cream of lime. Remove and wash any insoluble material. Dilute the aqueous solution to 400 ml. and reprecipitate the calcium salts with 3420 ml. of absolute ethanol. Filter off the precipitate and wash with ethanol.

4. Isolation of Glutamic Acid. Dissolve the precipitate in 800 ml. of hot water and wash any residue with small portions of hot water. Remove the calcium with a *very* slight excess of oxalic acid. Wash the precipitate of calcium oxalate. Concentrate the filtrate and washings to 80 ml. Remove any further quantity of $(COO)_2Ca$

---

* Best isolation method.

as well as Ca++ and (COOH)₂. Add 20 ml. of concentrated HCl and
evaporate the solution to a thick syrup *in vacuo*. Add a further
quantity of concentrated HCl or HCl gas. The glutamic acid will
crystallize out as the hydrochloride after standing for several days
in a cold place.

5. Purification of Glutamic Acid. Filter and wash the crystals
of glutamic acid hydrochloride on a sintered glass crucible and re-
crystallize the precipitate from 20 ml. of boiling water and 5 ml. of
concentrated HCl. Evaporate the hot solution to incipient crystal-
lization. Filter off the glutamic acid hydrochloride, wash it with
cold HCl and dry. Determine the purity of the compound by its
optical rotation in 9 per cent HCl (+32°), by nitrogen (7.64 per
cent) and titration of the salt in water to pH 7.3 (brom thymol
blue).

Glutamic Acid = 0.80×glutamic acid hydrochloride.

6. Copper Aspartate. Combine the mother liquors from the
glutamic acid experiments and evaporate to dryness to remove the
excess HCl. Dissolve the residue in boiling water and add an excess
of CuCO₃. Wash the precipitate thoroughly with hot water. Con-
centrate the filtrate and washings to incipient precipitation. Cool
several days in the refrigerator, remove and wash the copper
aspartate with water, alcohol, and ether. Dry the copper salt to
constant weight in a vacuum desiccator.

Aspartic Acid = 0.673×copper aspartate.

7. Purification of Copper Aspartate. If necessary, suspend the
copper salt in hot water and add HCl dropwise until all the pre-
cipitate dissolves. Then add an excess of CuCO₃ to the hot solution.
Filter off the excess CuCO₃ and wash it thoroughly with hot water.
Concentrate the filtrate and washings to incipient precipitation.
Cool for some days and remove the pure copper aspartate as de-
scribed above.

8. Isolation of Further Quantities of Dicarboxylic Acids. Acidify
the filtrates from the copper aspartate precipitations with HCl and
remove the copper with H₂S. Concentrate the solution to a small
volume and precipitate any dicarboxylic acids with calcium hydrox-
ide and alcohol. Small quantities of glutamic and aspartic acids can
be isolated from this precipitate by the methods given above.

Comment: Chibnall, Rees, Williams and Boyland (157) proved
that the use of Cu₂O or Cu₂Cl₂ to "clean up" protein hydrolysates
results in large losses of glutamic acid (contra *cf.* below).

Chibnall (158) says "The true values (by this general method)

must be between 1 and 2 per cent of the protein higher for glutamic acid and between 0.5 and 1 per cent (higher) for aspartic acid."

The Ritthausen-Foreman procedure as simplified by Chibnall has given excellent results in our hands. The quantities of alcohol suggested for the precipitation of the calcium salts can, at times, be reduced. We have found, following a suggestion of Dr. H. Waelsch, that the deeply colored mother liquor can be easily removed from the glutamic acid hydrochloride with cold acetone. The resulting product is colorless and usually almost analytically pure.

Professor R. Keith Cannan has informed us that the dicarboxylic amino acids are quantitatively removed from a protein hydrolysate made cation-free by the method of Block (107), by exchange on synthetic anion exchange substances. After thoroughly washing the "zeolite" with water to remove the non-acidic amino acids, the dicarboxylic acids are quantitatively removed either by strong alkalies or by exchange with mineral acids, cf. Block, 107.

Van Slyke et al. (636, 637) have suggested that the quantities of glutamic and aspartic acids present in mixtures in the absence of other amino acids can be calculated from the fact that oxidation with ninhydrin yields 1.0 mol of CO_2 with glutamic acid and 1.9 mols from aspartic acid.

Bailey, Chibnall, Rees, and Williams (158) have facilitated the isolation of pure copper aspartate by removing all the cystine and cystine decomposition products before precipitation of the calcium salts with alcohol. The hydrochloric acid hydrolysate, after distillation of the excess mineral acid by repeated concentration *in vacuo*, is warmed to 45 to 50° C and an aqueous suspension of cuprous oxide (*cf.* Chapter III, Part II, Section 5) is poured slowly into the rapidly stirred hydrolysate until a test sample shows an excess of Cu_2O to be present and the supernatant liquid is deep blue. The solution is stirred an hour longer and is placed at 0° over night. The insoluble precipitate is removed and is washed thoroughly with ice water.

PART III

1. The Estimation of Glutamic Acid by Conversion to Pyrrolidone Carboxylic Acid

A. Opsahl and Arnow's Method (486) based on the Experiments of Pucher (534) and of Wilson (680)

Principle: Wilson and Cannan (680) showed that in aqueous solutions, close to neutrality, equilibrium between glutamic acid and pyrrolidone carboxylic acid favors almost complete dehydration. While in strongly acid or alkaline solutions, the conversion of pyrrolidone carboxylic acid to glutamic acid is rapid and practically complete.

$$\text{HOOC·CH—NH}_2 \qquad \text{HOOC·CH——NH}$$
$$\text{CH}_2 \quad \rightleftarrows \quad \text{CH}_2 \qquad +\text{H}_2\text{O}$$
$$\text{CH}_2\text{COOH} \qquad \text{CH}_2\text{——CO}$$
$$\text{Glutamic Acid} \qquad \text{Pyrrolidone Carboxylic Acid}$$
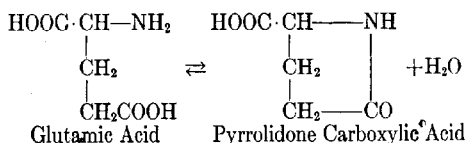
Pucher and Vickery (534) found that pyrrolidone carboxylic acid could be quantitatively removed from a dilute acid solution (pH 2.4 ± 0.2) by continuous extraction with ethyl acetate.

Method: 15 gm. of casein were hydrolyzed with 20 per cent HCl for 20 hours. The acid was removed by concentration *in vacuo* and the residue was taken up in 60 ml. of water. The solution was adjusted to pH 3.3 with 15 N NaOH and the glutamic acid was dehydrated by boiling under reflux for 50 hours. The solution was concentrated to 30 ml. and any precipitate was removed and washed with cold water.

The pH was now adjusted to 2.5 with HCl and the pyrrolidone carboxylic acid was extracted with ethyl acetate in a continuous extractor for 50 hours. The solvent was removed and the residue was dissolved in 40 ml. of 9 per cent HCl. The acid solution was boiled under reflux for 2 hours to convert the pyrrolidone carboxylic acid to glutamic acid.

The solution was then evaporated to 20 ml. and the glutamic acid was precipitated as the hydrochloride after saturation with gaseous HCl.

Comment: Although Wilson and Cannan (680) say that pre-

liminary experiments along this line (glutamic acid⇌pyrrolidone carboxylic acid) do not encourage the hope of developing a simple method for the separation or determination of glutamic acid, the results of Opsahl and Arnow (486) and of Woodward and Reinhart (688) suggest that this procedure may at times be used advantageously.

Woodward and Reinhart (688) increased the extraction of pyrrolidone carboxylic acid from water with ethyl acetate by adding 10 to 20 per cent of Na_2SO_4 to the water layer (salting out).

2. THE OXIDATION OF GLUTAMIC ACID TO SUCCINIC ACID
(ARHIMO, 34 AND COHEN, 167)

*A. The Micro-oxidation Procedure of Cohen and Krebs (167 and 390)

Principle: Glutamic acid is oxidized with chloramine T to β-cyanopropionic acid. The latter is hydrolyzed to succinic acid. Succinic acid is then determined manometrically according to Krebs (390) by measuring the oxygen consumption necessary to oxidize it to fumaric acid by succinic dehydrogenase.

$$COOH \cdot CH_2CH_2 \cdot COOH$$
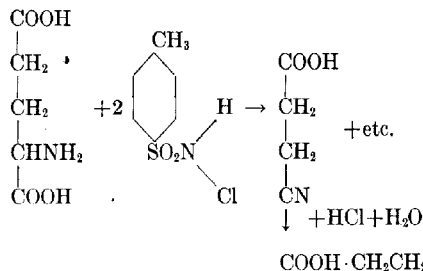
Reagents: Citrate buffer: Dissolve 17.65 gm. of $Na_3C_6H_5O_7 \cdot 2H_2O$ and 8.40 gm. of $C_6H_8O_7 \cdot H_2O$ in water and dilute to 50 ml.

0.1 M Phosphate buffer (*pH* 7.4): Dissolve 17.8 gm. of $Na_2HPO_4 \cdot 2 H_2O$ in 500 ml. of water, add 20 ml. of N HCl and dilute to 1 liter.

Chloramine T: Prepare a 10 per cent solution fresh each day.

Succinoxidase: Wash 50 gm. of minced pigeon muscle 3 times with 500 ml. portions of water on muslin. Suck the residue as dry as possible and then suspend it in 4 to 5 volumes of 0.1 M phosphate buffer. The enzyme keeps 7 to 10 days in the cold before extraction with phosphate.

Apparatus: The standard Warburg apparatus with conical cups

* Recommended procedure.

provided with side-arms and central chambers is used. The side-arm should hold 1 to 1.5 ml.

Method: 1. Oxidation. Adjust an aliquot of the protein hydrolysate to pH 4.7 with 1 to 1.5 ml. of citrate buffer and oxidize the glutamic acid with 2 ml. of chloramine T solution by shaking at 40° for 10 minutes. Then cool the solution in an ice bath and filter off the p-toluene sulfonamide. Wash the precipitate with ice water.

2. Extraction of Cyanopropionic Acid. Acidify the aqueous solution with 4 ml. of 10 per cent H_2SO_4 and extract the β-cyanopropionic acid with ether in a continuous extractor for 1 to 2 hours. Add 2.5 ml. of phosphate buffer to the etheral solution and distill off all the ether. Cool the solution and remove any further precipitate of p-$CH_3C_6H_4SO_2NH_2$.

3. Hydrolysis to Succinic Acid. Add concentrated HCl to the aqueous solution of the cyano acid until the concentration of mineral acid is 12.5 per cent. Place the solution in boiling water for 15 minutes. Cool and add concentrated NaOH, dropwise, until the solution becomes quite hot, add 0.5 ml. of 5 per cent ammonium chloride to destroy any chloramine T. Then add a few drops of phenol red and sufficient NaOH to make the solution purple. Extract the last traces of p-toluene sulfonamide from the alkaline solution with ether.

4. Extraction of Succinic Acid. Acidify the aqueous solution with 3 ml. of 10 per cent H_2SO_4 (light yellow to phenol red) and extract the succinic acid with ether. Add 2 to 3 ml. of $M/10$ phosphate buffer to the ether layer and slowly distill off the ether. Concentrate the aqueous solution to 1 ml. to remove all traces of ether.

5. Determination of Succinic Acid. Adjust the solution of succinic acid to pH 7.4 with phosphate buffer. Place 4 ml. of enzyme preparation into the main part of the cup. Then introduce 0.5 to 1.5 ml. of succinic acid solution into the side-arm and 0.2 ml. of 2 N NaOH into the center chamber. Measure the oxygen consumption as usual, subtract the oxygen uptake due to the enzyme alone.

$$100 \text{ uL of } O_2 \doteqdot 1.05 \text{ mg. of Succinic Acid.}$$

Comment: Recoveries of succinic acid of 93 to 99 per cent have been achieved by this manometric procedure (Krebs, 390; Woodward and Reinhart, 688).

Bovarnick (116) advises hydrolysis of the cyanopropionic acid with 20 per cent sulfuric acid for 3 to 4 hours. The second ether extraction may be omitted without the introduction of any error.

B. Woodward, Reinhart, and Dohan's Modification of the Cohen-Krebs Method (687)

Procedure: 60 mg. of a protein hydrolysate in 3 ml. of solution are adjusted to p_H 4.7 with concentrated NaOH. Brom cresol green is used as the internal indicator. Then 1.5 ml. of 2 M citrate buffer of p_H 4.7 and 3 ml. of freshly prepared 10 per cent aqueous chloramine T are introduced. The solution is shaken at 40° for 10 minutes and then cooled in an icebath for 15 minutes. The precipitate of p-$CH_3C_6H_4SO_2NH_2$ is filtered off and washed with 4 ml. of ice water. The volume is adjusted to 11.5 ml. Then 6.4 ml. of concentrated HCl are added and the β-cyanopropionic acid is hydrolyzed to succinic acid by heating at 100° for 15 minutes.

The remainder of the procedure is according to Cohen and Krebs (167, 390).

Comment: There are a number of micro methods available for the determination of succinic acid; that of Goepfert (259) appears simpler than Pucher and Vickery's procedure (535).

Goepfert's Micro-Determination of Succinic Acid (259). The solution of organic acids is acidified to Congo paper with H_2SO_4 and heated on the steam bath. $N/10$ $KMnO_4$ is added to the hot solution until a brown precipitate is obtained. The precipitate of MnO_2 is removed with Na_2SO_3 and the solution is evaporated to dryness. The residue is taken up in 15 ml. of water and 2 ml. of concentrated H_2SO_4 are added. The solution is saturated with K_2SO_4. The aqueous solution is transferred into a liquid-liquid continuous extractor by the aid of 4, 3, and 3 ml. portions of a saturated solution of K_2SO_4. The solution is extracted with ether for 4 hours or longer. The ethereal solution is removed and 5 ml. of water are added. The ether is distilled off and the final traces are removed from the water by boiling for 5 minutes. One drop of a 0.3 per cent aqueous solution of m-nitro-phenol is added and then sufficient 0.05 N NaOH to turn the solution yellow. The solution is then decolorized by the addition of 1 drop of 0.1 N HNO_3 and a calculated excess of 0.02 M $AgNO_3$ and 1 drop of 0.05 N NH_4OH (to bring the solution to neutrality) are added. The precipitate is allowed to form in the dark for 2 hours, after which it is removed by filtration through a Gooch or sintered glass crucible. The precipitate is thoroughly washed with 3 portions of 1 per cent NH_4NO_3. Then 2 drops of dichlorofluorescorcin (0.1 per cent in 70 per cent ethanol) and 7 to 9 drops of 1 per cent chloride-free starch are added. The excess silver is titrated with 0.02 M KBr until the pink color disappears.

1 ml. of 0.02 M $AgNO_3$ $\approx$1.18 mg. of Succinic Acid.

C. The Oxidation Method of Arhimo and Laine (34)

Principle: Glutamic acid is deaminated to hydroxyglutaric acid. The latter is oxidized with $KMnO_4$ to succinic acid which is determined quantitatively.

Procedure: 1. Precipitation of Glutamic Acid. The dicarboxylic amino acids are separated from the majority of the other amino acids by carrying out a Ritthausen-Foreman calcium salt separation on one gram or less of hydrolyzed protein.

2. Deamination. 1 ml. of 2 N sulfuric acid and 2 ml. of 30 per cent $NaNO_2$ are added to 5 ml. of the calcium-free Foreman precipitate containing 1 to 10 mg. of glutamic acid. The solution is shaken for 10 minutes and the excess HONO is destroyed by boiling.

3. Oxidation. The solution is cooled to room temperature and the hydroxy acids are oxidized with an excess of 1.5 N $KMnO_4$. After 1 hour, the precipitate is removed and the succinic acid is extracted with freshly distilled ether in a continuous extractor for 48 hours.

4. Determination. The ethereal solution is evaporated to dryness and the residue is dissolved in ethanol. An excess of a saturated alcoholic solution of silver nitrate is added and the precipitate of silver succinate is removed and washed with alcohol. The precipitate is dissolved in dilute HNO_3 and the liberated silver nitrate is titrated with N/200 NH_4SCN using Mohr's salt as the indicator.

1 ml. of N/200 NH_4SCN $\approx$0.368 mg. of Glutamic Acid.

Comment: If 25 to 500 mg. of glutamic acid are available, the silver succinate can be weighed.

Glutamic Acid = 0.443×silver succinate.

PART IV

1. The Estimation of Aspartic Acid by Oxidation and Bromination (Arhimo, 33)

Principle: Aspartic acid, free from tyrosine, is treated with bromine and potassium permanganate according to Pucher *et al.* (532). The resulting compound, dibromoxalacetic acid (?), is then estimated colorimetrically.

$$\begin{array}{l} CHNH_2COOH \\ | \\ CH_2COOH \end{array} + Br_2 + KMnO_4 \rightarrow \begin{array}{l} COOH \\ | \\ COCBr_2COOH \end{array} (?)$$
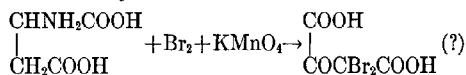
Reagents: M KBr:11.9 gm. of salt for 100 ml. of water.

Petroleum Ether: boiling range 35 to 40°C.

Dinitrophenylhydrazine: 5 gm. of commercial dinitrophenylhydrazine are dissolved in 200 ml. of concentrated HCl and 800 ml. of water. The solution is boiled 1 to 2 minutes, stirred, and diluted to 1000 ml. The solution should be filtered before use.

Hydrogen Peroxide: Halogen-free peroxide is prepared by weakly acidifying 50 ml. of an 8 per cent solution of Na_2O_2 with dilute H_2SO_4.

Method: 1. Precipitation of Aspartic Acid. The aspartic acid is precipitated from a protein hydrolysate by the Ritthausen-Foreman method.

2. Oxidation. An aliquot of solution, containing 0.2 to 2.0 mg. of aspartic acid, is diluted with water to 20 ml. Then 3 ml. of 1:1 H_2SO_4 are added and the solution is boiled gently for 8 to 10 minutes. After cooling, 1 ml. of a saturated aqueous solution of bromine is added. The reaction is allowed to continue at room temperature for 5 minutes. The solution is filtered and the paper is washed with water. (Volume = 35 ml.) Then 2 ml. of M KBr and 5 ml. of 1.5 N KMnO$_4$ are added. The reaction is allowed to take place at 20 to 22° for 10 minutes. The solution is then cooled to 5 to 10° and decolorized with 3 per cent H_2O_2 added dropwise.

3. Separation of Pentabromacetone. Any CBr$_3$COCHBr$_2$ which may have been formed from citric acid is removed by extraction with petroleum ether.

4. Color Development. The aqueous solution is diluted to 100

ml. A 25 ml. aliquot of this solution is placed in a 100 ml. Kjeldahl flask together with 25 ml. of water and 0.5 ml. of 1.5 N $KMnO_4$. The solution is then decolorized with 2 ml. of 4 per cent Na_2S (freshly prepared). 40 to 45 ml. of solution are then distilled, using an apparatus similar to that described by Folin and Wright (229), into 10 ml. of dinitrophenylhydrazine plus 20 ml. of water. The distillate is cooled and the precipitate is filtered on a Gooch crucible and washed with water. The precipitate is dissolved in boiling pyridine and the solution is diluted to 25 ml.

50 ml. of water and 5 ml. of 5 N NaOH are added to a 2 to 5 ml. aliquot of the pyridine solution, and the mixture is diluted to 100 ml. The colored solution is read in a colorimeter using filter 570 mu. Aspartic acid is used as the standard.

Comment: The yield of the brom compound is influenced by the temperature at which the $KMnO_4$ oxidation is carried out and by the quantity of KBr used.

Separation of the dicarboxylic amino acids from the monoamino acids can be readily achieved with synthetic ion exchangers (*cf.* Cannan, 148).

2. The Conversion of Aspartic Acid to Fumaric Acid (Dakin, 190)

Principle: Aspartic acid is converted to fumaric acid by the following reactions:

$$
\begin{array}{c}
\text{COOH} \\
| \\
\text{CHNH}_2 \\
| \\
\text{CH}_2 \\
| \\
\text{COOH}
\end{array}
\xrightarrow[\text{NaOH}]{(\text{CH}_3)_2\text{SO}_4}
\begin{array}{c}
\text{CO}-\text{O} \\
| \quad | \\
\text{CH}-\text{N}(\text{CH}_3)_3 \\
| \\
\text{CH}_2 \\
| \\
\text{COOH}
\end{array}
\xrightarrow{\text{H}_2\text{SO}_4}
\begin{array}{c}
\text{HOOC}\cdot\text{CH} \\
\| \\
\text{HC}\cdot\text{COOH}
\end{array}
$$
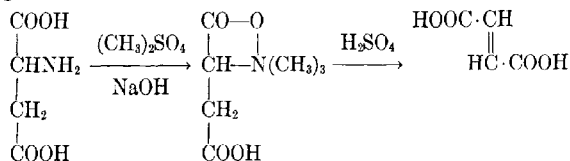
The fumaric acid is separated from the amino acids by ether extraction.

Method: An aliquot of a protein hydrolysate is treated with an excess of dimethylsulfate and the solution is kept alkaline for 1 hour by the repeated addition of small quantities of 33 per cent NaOH. The solution is allowed to stand for 2 hours until it becomes feebly acid. Then, the reaction mixture is acidified with dilute H_2SO_4 and the fumaric acid is removed by extraction with ether. The ether is distilled off and the fumaric acid is weighed. M.P. is 287 to 290° in a closed capillary.

Comment: Further results on this method, based on the procedure of Engeland (210), are awaited with interest.

3. Miscellaneous Suggestions for the Estimation of Aspartic Acid

Eegriwe (205) has found that malic acid, prepared by the deamination of aspartic acid, will condense with β-naphthol on warming in 96 per cent H_2SO_4 to give a yellow colored compound with a bluish fluorescence. Glycollic acid from glycine, which interferes, can be removed by precipitation of the aspartic acid by the Ritthausen-Foreman calcium salt procedures or synthetic ion exchange substances or the malic acid can be separated, after deamination, by precipitating it in neutral reaction with a slight excess of a saturated solution of basic lead acetate (McChesney, 445).

Fromageot and Heitz (245) have suggested that the sum of serine, alanine, and aspartic acid can be estimated after deamination by oxidizing their respective hydroxy acids to acetaldehyde with $KMnO_4$ and $MnSO_4$. Aspartic acid can be estimated separately by this procedure if it is originally separated from the monoamino acids.

CHAPTER VI

PART V

ANALYTICAL RESULTS

As in the previous chapters, the values given for glutamic and aspartic acids have been calculated to 16.0 per cent of nitrogen. The great majority of the values given in these tables represent minimal figures. In the case of the older analyses, the results for glutamic acid are probably 25 to 50 per cent too low, while those for aspartic acid are probably only half of the values which would be obtained by the best modern methods. These facts should be borne in mind when physico-chemical calculations are made using the older data.

Dicarboxylic Amino Acids in *Animal Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	GLUTAMIC ACID	ASPARTIC ACID
				per cent	gm.	gm.
Albuminoids:						
Gelatin	Foreman	Dakin	185	18.0	5.2	3.0
Gelatin	Foreman	Kingston	365		5.4	8.5
Collagen		Theis	604	(16.0)	5.8	3.4
Elastin	Foreman	Stein	586	17.1	2.5	0.0
Blood Proteins:						
Fibrin	Foreman	Bergmann	67	17.7	12.8	5.3
Fibrin	Isotope	Rittenberg	547	15.2	13.8±0.1*	11.9*
Hemoglobin	Foreman	Bergmann	67	17.0	3.3	6.0
Hemoglobin-Beef	Foreman	Chibnall	158	(16.7)	5.6*	7.7*
Hemoglobin-Horse	Foreman	Chibnall	158	(16.7)	5.8*	8.4*
Seroglobulin	Foreman	Calvery	145	16.0	6.8	4.7
Bence-Jones	Fischer	Abderhalden	11	(16.0)	6.0	4.5
Bence-Jones	Foreman	Calvery	143	18.0	8.6*	4.7*
Bence-Jones	Hlasiwetz, Fischer	Hopkins	308	16.2	7.0	2.1
Egg Proteins:						
Egg Albumin	Foreman	Chibnall	160	15.8	16.3*	8.2*
Egg Albumin	Foreman	Jones	343	15.2	14.0	6.5
Egg Albumin	Fischer	Osborne	497	15.5	9.4	2.3
Livetin	Foreman	Jukes	348	15.5	7.0	3.1
Vitellin	Hlasiwetz	Abderhalden	13	(16.3)	12.2	
Vitellin	Hlasiwetz, Fischer	Osborne	495	16.3	12.7	2.1
Hormones, Enzymes:						
Pepsin	Foreman	Calvery	146	15.4	18.9	6.8
Secretin	Foreman	Ågren	26	(16.0)	6.4ᵃ	
Insulin	Foreman	Jensen	320	15.5	21	

Dicarboxylic Amino Acids in *Animal Proteins* (Continued)

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITROGEN	GLUTAMIC ACID	ASPARTIC ACID
			per cent	gm.	gm.
Keratins:					
Wool	Hlaziwetz, Fischer	Abderhalden 16	16.6	12.3	2.2
Wool	Foreman	Gordon 261		10.1	5.5
Wool	Foreman	Speakman 584	(16.0)	15.3*	7.3*
Hair-Horse	Fischer	Abderhalden 9	(16.0)	3.7	
Hair-Cow	Foreman	Block 109	15.5	12.2*	3.0*
Feathers-Goose	Fischer	Abderhalden 10	(16.0)	2.3	
Feathers-Gull	Foreman	Speakman 584	(16.0)	9.7*	6.6*
Horn-Cattle	Hlaziwetz, Fischer	Abderhalden 16	15.1	18.2	2.7
Horn-Cattle	Hlaziwetz	Fischer 224		15	
Scyllium	Fischer	Pregl 529	15.1	7.6	2.4
Egg Membrane	Fischer	Abderhalden 14	(16.0)	8.1	1.1
Egg Membrane	Foreman	Calvery 142	16.6	9.7*	3.3*
Spongin	Fischer	Abderhalden 12	(16.0)	18.1	4.7
Spongin	Fischer	Clancy 163		18.4	4.5
Liver Proteins:					
Carcinoma	Foreman	Chibnall 157	16.2	10.6	6.9
Rat	Hlaziwetz (?)	Johnson 336	(16.0) [a]	12.2	
Milk Proteins:					
Casein	Foreman	Chibnall 158	15.6	21.3	6.3*
Casein	Dakin	Dakin 190	(15.6)		5.0
Casein	Foreman	Foreman 239	15.6	24.2*	1.8
Casein	Foreman	Foreman 240	15.6	22.3	1.8
Casein	Foreman-Ca(OH)₂	Opsahl 486	15.5	16.9	
Casein	Foreman-Ba(OH)₂	Opsahl 486	15.5	23.2	
Casein	Dakin-Foreman	Opsahl 486	15.5	12.7	
Casein	Pucher-Wilson	Opsahl 486	15.5	19.6	
Casein	Hlaziwetz	Osborne 488	(15.6)	11.2	
Casein	Hlaziwetz	Osborne 502	15.6	16.1	
Casein-Human	Hlaziwetz, Fischer	Abderhalden 17	(15.5)	11.0	6.3
Lactalbumin	Foreman	Jones 340	15.4	13.4	9.7
β-Lactoglobulin	Foreman	Chibnall 160	15.6	22.1*	10.1*
Muscle Proteins:					
Cod	Fischer	Abderhalden 24	13.6	8.8	0.7
Ox	Foreman	Jones 343	16.2	13.5	6.0*
Ox	Fischer	Osborne 498	16.2	15.4*	4.1
Herring		Wakamatu 669	12.5	12.7	4.5
Halibut	Foreman	Jones 343	16.5	13.3	7.8
Fish	Fischer	Osborne 493	(16.0)	10.1	2.7
Chicken	Fischer	Osborne 493	(16.0)	16.5	3.2
Scallop	Hlaziwetz, Fischer	Osborne 496	17.1	14.1	3.3
Myosin	Foreman	Sharp 575		21.0*	8.5*
Tissues:					
Heart	Foreman	Chibnall 157	14.3	13.3*	6.9*
Lung	Foreman	Chibnall 157	15.3	9.3	
Lens	Fischer	Hijikata 298	(16.0)	15.5	
Mouse-Sarcoma	Foreman	Chibnall 157	15.0	8.9	
Human-Tumors	Isotope	Graff 265		11.3–14.6*	
Tumors	Cohen-Krebs	Woodward 687	(16.0)	6–12	

* "Best Values."

[a] Includes Aspartic Acid.

Dicarboxylic Amino Acids in *Plant Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	GLUTAMIC ACID	ASPARTIC ACID
				per cent	gm.	gm.
Autotropic Plants:						
Phormidium	Foreman	Mazur	442		7.4	1.4
Ulva	Foreman	Mazur	442		12.8	6.2
Laminaria	Foreman	Mazur	442		12.3	2.9
Sargassum	Foreman	Mazur	442		4.9	9.0
Chondrus	Foreman	Mazur	442		13.8	3.8
Biologically Active:						
Cytochrome C	Foreman-Nitrogen	Theorell	605	15.4	16.4	5.3
Cytochrome C	Foreman-Isolation	Theorell	605	15.4	6.2	1.7
Yellow Enzyme	Foreman	Kuhn	393	16.3	6.8	
Allergen-Cottonseed	Hlaziwetz, Foreman	Spies	585	19.8	11.5	
Allergen-Cottonseed	Hlaziwetz, Foreman	Spies	585	11.6	15.7	
Ricin	Fischer	Karrer	555	(17.0)	19.0	1.9
Tobacco Virus	Foreman	Ross	557	15.9	6.0	3.0
Tyrocidine	Chromatographic	Gordon	261E		10–12	9.3–10.5
Corn Proteins:						
Zein	Brazier	Brazier	129	(16.1)	35.6*	3.2
Zein	Foreman	Dakin	186	16.1	31.3	1.8
Zein	Arhimo	Laine	398	(16.1)	30.9	3.4*
Zein	Hlaziwetz, Fischer	Osborne	490	16.1	18.3	1.4
Zein	Hlaziwetz, Fischer	Osborne	499	16.1	26.5	1.7
Gluten	Colen-Krebs	Reinhart[a]			24.5±.4	
Leaf Proteins:						
Cocksfoot	Foreman	Miller	452		13.1	5.3
Miscellaneous:						
Cottonseed Globulin	Hlaziwetz	Abderhalden	6	unc.	17.2	
Coconut Globulin	Hlaziwetz, Daken	Johns	334	18.5	16.6	4.5
Coconut Globulin	Hlaziwetz, Foreman	Jones	339	18.5	16.2	3.4
Arachin	Foreman	Johns	322	18.3	14.6	4.6
Arachin	Foreman	Jones	343	18.3	17.0*	4.9*
Edestin	Hlaziwetz	Abderhalden	3	unc.	6.3	
Edestin	Foreman	Gordon	261		17.5	10.3
Edestin	Foreman	Jones	343	18.8	16.3	8.7
Edestin	Foreman	Chibnall	160	18.7	17.8*	10.3*
Glycinin	Foreman	Jones	343	17.0	17.4	8.8
Soy Bean Meal	?	Heinrich	286	?	19.1	3.7
Lupin	?	Heinrich	286	?	27.2	5.4
Rubber Latex	Foreman	Tristram	620	15.0	12.9	9.9
Hordein	Hlaziwetz, Fischer	Kleinschmitt	369	17.2	38.4	1.2
Mold	Foreman	Woolley	689	5.15	+	+
Wheat Proteins:						
Gliadin	Hlaziwetz	Abderhalden	7	unc.	31.5	
Gliadin	Foreman	Chibnall	157	17.8	40.7	
Gliadin	Foreman	Chibnall	158	(17.8)	42.2*	1.3*
Gliadin	Foreman	Jones	343	17.9	38.4	0.7
Gliadin	Hlaziwetz, Fischer	Osborne	503	(17.7)	46	
Gliadin	Hlaziwetz	Osborne	489	17.7	33.7	
Glutenin	Foreman	Jones	343	16.8	24.7	1.9
Leucosin	Hlaziwetz	Osborne	489	16.8	6.4	3.2
Gluten		Padoa	508		26.8	9.6

* "Best Values."
[a] Personal Communication.

GLYCINE AND ALANINE

	Glycine	Alanine
Empirical Formula	$C_2H_5O_2N$	$C_3H_7O_2N$
Optical Form		d
Molecular Weight	75.05	89.07
Carbon	31.98	40.42
Hydrogen	6.71	7.93
Nitrogen	18.67	15.73
Oxygen	42.64	35.92
Melting Point	225–230°	297° (decomp.)

PART I

THE ESTIMATION OF GLYCINE

1. THE ISOLATION OF GLYCINE

A. Isolation as Glycine Ester Hydrochloride (Fischer, 223)

HISTORICAL: Fischer (223) reported in 1902 that glycine could be recovered in good yield (79 per cent) by direct precipitation from a casein hydrolysate as the ester hydrochloride. This procedure is useful for preparative purposes but not for quantitative analysis.

B. Isolation by the Ester Distillation Method (220)

Historical: Fischer, Osborne, and their coworkers found that a portion of the glycine which did not precipitate from the amino acid mixture as the ester hydrochloride could be distilled in the lower boiling fractions by the ester distillation method. Separation from the lower boiling ester fractions was from then on rather generally employed to estimate the quantities of glycine in protein hydrolysates. Levene (414) showed that if an excess of picric acid were added to the mixture of glycine and alanine, obtained from the lowest boiling ester fraction, the glycine would precipitate as the picrate provided that not too much alanine was present. The glycine picrate melted at 190° after recrystallization from water.

In 1909, Osborne and Jones (498) isolated 1.9 gms. of glycine from the lowest boiling ester fraction (100° bath temperature, 18 mm. pressure) from 500 gm. of ox muscle hydrolysate, but they found 8.5 gm. of glycine in the ether distilled off at atmospheric

pressure from the amino acid ester mixture. It had been the usual practice to discard the ether distillates because no one believed that the relatively high boiling amino acid esters could come over at 35° and 760 mm. pressure. The following year, Osborne and Liddle (500) isolated considerable portions of glycine, alanine, and leucines by acidification of the ether distillate. They said "These results show that a not inconsiderable loss of amino acids has occurred in past analyses of proteins, through neglect to recover that part of them carried over with the ether." This finding added to the other known difficulties of the ester method makes the values for glycine and alanine estimated by this procedure of qualitative interest only.

C. Isolation of Glycine Carbamate (Kingston and Schryver, 365)

Principle: Glycine carbamate is insoluble in cold water.

Method: The protein is hydrolyzed with 25 per cent H_2SO_4 and the mineral acid is removed by $Ba(OH)_2$. The $BaSO_4$ is then removed and washed. Sufficient $Ba(OH)_2$ is added to react with all the carboxyl groups as estimated by formol titration on a small aliquot of the hydrolysate. Then 2 to 3 volumes of ethanol are added to the solution and the barium salts of glutamic and aspartic acids are filtered off after standing several days.

Powdered $Ba(OH)_2 \cdot 8H_2O$ is then added to the flltrate, during constant stirring, until the solution is strongly alkaline to phenolphthalein. A stream of CO_2 is passed into the solution to neutral to phenolphthalein. Then more powdered baryta is introduced, followed by CO_2. This process is repeated a number of times until no more N is precipitated. The reactions are conducted at 0° throughout. The barium carbamates are filtered off and washed with alcohol and ether. All the barium carbamates except that of glycine are soluble in ice cold water. The carbamates are decomposed by boiling in water.

Comment: Dakin (185) found some glycine may be precipitated as the barium salt along with glutamic and aspartic acids in the Ritthausen-Foreman method.

D. Isolation of Glycine as the Trioxalatochromiate (Bergmann and Fox, 64)

Principle: In 1935, Bergmann and Fox (64) showed that glycine formed insoluble salts in dilute acid solution with potassium trioxalatochromiate. The latter reagent appears to have been prepared accidentally by Wilton Turner in 1830 (*cf.* 174) and has been studied by Croft in 1842 (174) and by Lapraik (403) in 1893.

Reagents: Potassium trioxalatochromiate (Croft, 174; Lapraik,

403). 19 parts of $K_2Cr_2O_7$ are added to a hot saturated solution of 23 parts of potassium oxalate and 55 parts of crystalline oxalic acid. As soon as the action ceases, the solution is evaporated to dryness. The residue is then taken up in water and the product is allowed to crystallize out in a flat glass dish. The salt is recrystallized if necessary.

Method: (largely based on Bergmann's experiments). 5 to 10 gm. of protein are hydrolyzed over night with 1:1 HCl. The excess acid is distilled off *in vacuo* and the hydrolysate is made alkaline to phenolphthalein with KOH. Alcohol is added and the ammonia is removed by vacuum distillation. The solution is acidified to approximately pH 2 with HCl and is decolorized with charcoal.

The filtrate and washings are concentrated to 50 ml. Two, 3, 4, 5, 6, 7, 8, and 9 ml. aliquots of the hydrolysate are pipetted into 125 ml. Erlenmeyer flasks. Sufficient N/10, N/5, N/2, and N HCl is added to each flask so that the final volume in each case is 10 ml. and the concentration of HCl is approximately N/10. Three gm. of K trioxalatochromiate are added to each flask and the flasks are shaken for 10 minutes to dissolve the salt. At the end of this time, 20 ml. of absolute ethanol are added and the flasks are shaken on the machine for 6 to 8 hours. After standing in the refrigerator over night, the precipitates are filtered off on #3 sintered glass crucibles and washed three times with 10 ml. portions of cold alcoholic HCl (2:1). The precipitates are dried in air and then dissolved in water. The aqueous solutions are diluted to volume and aliquots are removed for nitrogen determinations. The glycine content of the protein is calculated from those experiments which give the maximum precipitation of nitrogen.

$$\text{mg. of Glycine} = 5.36 \times \text{mg. of Nitrogen}$$

Comment: Bergmann (64, 69) originally claimed that glycine alone, of all the constituents usually present in a protein hydrolysate, was precipitated by K trioxalatochromiate. Later, Bergmann and Stein (70) found that this was not true and that some ammonia was also precipitated.

If it is not desirable to remove the NH_3 from the original protein hydrolysate, the glycine precipitated can be estimated by the difference in total N and ammonia N.

Bergmann and Niemann (69) claim that 88 ± 1 per cent of the glycine in a protein hydrolysate can be precipitated under the optimal conditions by this method. Their experiments were, however, carried out before they were cognizant of the ammonia error.

E. The Isolation of Glycine as the Nitranilate (Town, 617)

Principle: In 1936, Town (617) claimed that glycine was specifically precipitated from an ammonia-free protein hydrolysate by nitranilic acid, 2,5-dihydroxy-3,6-dinitro-p-benzoquinone, in the presence of an excess of alcohol.

Method: Remove the NH_3 from 200 to 300 mg. of a protein hydrolysate with baryta. Then precipitate the barium with a slight excess of H_2SO_4. Remove the $BaSO_4$ and concentrate the filtrate and washings to 4 ml. Add 30 ml. of absolute alcohol and remove any further precipitate of $BaSO_4$. Then add 5 ml. of a freshly prepared solution of 300 mg. of nitranilic acid in absolute alcohol. Stand over night, filter and weigh the precipitate of glycine nitranilate.

Comment: Stein and Miller (586) showed that 82 per cent of the histidine present in an amino acid mixture was precipitated along with the glycine. The method is, therefore, of value only in the absence of histidine (and lysine). However, Town's use of nitranilic acid was responsible for the best isolation method for histidine (*cf.* Chapter I).

2. THE COLORIMETRIC ESTIMATION OF GLYCINE

A. Zimmermann's o-Phthaldialdehyde Reaction (697)

Principle: Glycine gives a violet color with o-phthaldialdehyde.
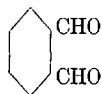
Reagent: Zimmermann's Method (697). Reflux 10 gm. of tetrabromo-o-xylene with 9 gm. of K oxalate, 62 ml. of water and 62 ml. of 95 per cent ethanol for 40 hours. At the end of this time, distill off 50 ml. of ethanol, and add 10 gm. of Na_3PO_4 and 300 ml. of water to the residue in the distillation flask. Then distill off 250 to 300 ml. of water. The distillate is preserved in a dark bottle away from sunlight.

Sandstrom and Lillivik's Procedure (564): Set up a 1 liter 3 necked flask with $CaCl_2$ drying tube, dropping funnel, and stirrer. Cool the flask in ice and add 150 ml. of acetic anhydride, 10 gm. of o-xylene, and 8 ml. of concentrated H_2SO_4. Then dissolve 26 gm. of CrO_3 in a mixture of 50 ml. of acetic anhydride and 60 ml. of glacial acetic acid. Add the chromic acid drop by drop with stirring. Keep the reaction flask in an ice bath for 4 to 5 hours. Stir the contents of the flask constantly. Pour the reaction mixture into a 1 liter beaker, one-quarter full of cracked ice. Keep at 4°C. over night.

Extract the oil with ether, wash the ether layer with water, and dry it over Na_2SO_4. Remove the ether by distillation.

The residue consists of acetic acid and phthaldialdehyde tetra-acetate. Add 50 ml. of 10 per cent H_2SO_4 to this residue and steam distill as long as a few drops of the distillate give a blue color with NH_4OH and CH_3COOH. This reaction yields approximately 500 ml. of glycine reagent. Store in a dark bottle and bring to pH 7.4 to 7.8 with phosphate buffer before use.

Chaudhuri's Method (154):
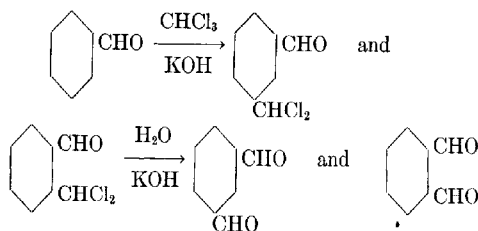
Reflux a mixture of 10 ml. of pure C_6H_5CHO, 8 ml. of $CHCl_3$, and 30 gm. of KOH in 50 ml. of water. After the reaction ceases, shake and then heat in an oil bath at 140–150° for 6 hours. Distill off the excess benzaldehyde and chloroform. Wash the residue with water several times and fractionally distill the chloraldehydes *in vacuo*.

o-Dichloromethylbenzaldehyde distills at 170–175° and m-dichloromethylbenzaldehyde, the larger fraction, comes over at 192–196°.

Hydrolyze the o-dichloromethylbenzaldehyde with aqueous KOH for 40 to 45 minutes. Cool and acidify the solution with HCl. o-phthaldialdehyde crystallizes out on standing in the cold.

Method: Add 10 drops of 2 N NaOH and 8 drops of phthaldialdehyde reagent to a neutral 1 per cent solution of glycine, shake 10 seconds and then acidify the solution with 10 drops of concentrated HCl. A violet color develops.

Comment: Ammonia, histidine, cysteine, tryptophane, and arginine disturb the test (Zimmermann, 697; Abderhalden, 22).

B. Klein and Linser's Modification of Zimmermann's Test (368)

Principle: The only colored compounds of o-phthaldialdehyde, besides that due to glycine, which are soluble in $CHCl_3$, are formed from ammonia and tryptophane. These two substances can be readily removed from protein hydrolysates.

Method: A. Macro. To be used with 0.5 mg. of glycine. Add to 10 ml. of a neutralized, tryptophane and ammonia-free, protein

hydrolysate, 15 ml. of a freshly prepared mixture of 25 parts of
$M/15$ phosphate buffer of pH 8.0 and 75 parts of the Zimmermann
o-phthaldialdehyde reagent. Shake for exactly 2 minutes. Then add
35 ml. of a freshly prepared mixture of 5 parts of concentrated
H_2SO_4 and 30 parts of 95 per cent ethanol. Shake for 2 minutes.
Introduce 30 ml. of chloroform, shake thoroughly and separate the
$CHCl_3$ layer. Add 1 ml. of ethyl alcohol for each 5 ml. of $CHCl_3$
solution. Read the color in a colorimeter against a glycine standard.
Use a light filter of 570 mu.

B. Micro. To be used with 0.05 mg. of glycine. To 0.5 ml. of the
neutralized glycine solution, add 0.75 ml. of buffered reagent. Shake
for 2 minutes and then add 1 ml. of alcoholic H_2SO_4. Shake for 2
minutes and extract the colored compound with 5 ml. of $CHCl_3$.
Remove 3 ml. of the $CHCl_3$ layer with a pipette and add 0.5 ml. of
alcohol. Read the violet color.

Comment: Klein and Linser (368) stress the point that all condi-
tions must be kept constant as the amount of color formed varies
with the quantity of o-phthaldialdehyde reagent used, etc. They
suggest removing the NH_3 from a protein hydrolysate by distilla-
tion from an alkaline, NaOH, solution *in vacuo*. The tryptophane is
precipitated with mercuric sulfate (*cf.* Chapter II).

C. Patton's Adaptation of the Zimmermann-Klein Method (511)

Method: 1. Hydrolysis, Destruction of Tryptophane and Re-
moval of Ammonia. Boil 3 gm. of protein with 50 ml. of 1:1 HCl
until complete solution. Then add 1 ml. of C_6H_5CHO and hydrolyze
for 24 hours longer. Concentrate the hydrolysate *in vacuo* to re-
move the excess HCl and C_6H_5CHO. Dilute the residue with water
and alkalinize the hydrolysate with NaOH or $NaHCO_3$. Remove
the NH_3 by distillation *in vacuo*. Neutralize the hydrolysate to
pH 6 to 8 and concentrate the solution to 10 ml. Remove any pre-
cipitate and wash it with 70 per cent ethanol. Dilute the hydroly-
sate to 100 ml.

2. Color Development. To 5 ml. of the tryptophane and am-
monia-free protein hydrolysate, add, in immediate succession, 2
ml. of $M/15$ phosphate buffer of pH 8.0 and 5 ml. of the o-phthal-
dialdehyde reagent. Mix after each addition and stand for 2 min-
utes. Then introduce 5 ml. of a freshly prepared, cooled mixture of
60 ml. of ethanol and 10 ml. of H_2SO_4. Mix and extract the color
with 10 ml. $CHCl_3$ by shaking for exactly 30 seconds. Separate and
remove 5 ml. of the chloroform solution by means of a dry pipette.
Add 1 ml. of ethanol to the $CHCl_3$ solution and shake the mixture
until the turbidity disappears. Compare the color with a glycine

standard prepared in the same way. A glycine-free protein hydrol-
ysate such as one prepared from zein is recommended as the re-
agent blank. Use light filter 560 mu.

Comment: The Zimmermann method, according to Klein and
Linser and to Patton, has given rather satisfactory results in the
authors' hands.

The addition of benzaldehyde to the hydrolysate is usually un-
necessary.

3. MISCELLANEOUS METHODS FOR GLYCINE

Comment: Rapoport (537) has described an oxidation method for
the estimation of the sum of serine and glycine while Abder-
halden (24) observed that glycine gives a blue violet color when
treated in alkaline solution with pyrocatechol, o-dihydroxyben-
zene.

CHAPTER VII

PART II

THE ESTIMATION OF ALANINE

1. THE ISOLATION OF ALANINE

Comment: The isolation of alanine from the lower boiling amino acid esters by the phosphotungstic acid precipitation method of Levene and Van Slyke (417) has been described in Chapter V, Section 1.

In 1937, Bergmann and Niemann (68, 69) described a procedure for the isolation of alanine as an oxalatochromiate after the removal of glycine with potassium trioxalatochromiate. Their rather involved procedure was applied only to silk fibroin which, as is generally known, contains an unusually large quantity of alanine. Bergmann's dioxpyridate procedure has been rarely employed and a further detailed description of the general method is awaited.

2. CALCULATION OF ALANINE FROM ACETALDEHYDE

A. *The Method of Kendall and Friedemann (358)*

Principle: Alanine is deaminated to lactic acid and the latter is determined quantitatively from the yield of acetaldehyde.

Procedure: Dissolve 10 to 50 mg. of alanine and 500 mg. of $NaHSO_4$ in 75 ml. of water and place the solution in a boiling water bath. Add, from a dropping funnel, 15 ml. of 2.5 per cent $NaNO_2$ at the rate of 1 ml. per minute. Then introduce in the same way, 15 ml. of 7.5 per cent of urea. Rinse and if necessary precipitate the carbohydrates with 20 ml. of a 20 per cent suspension of $Ca(OH)_2$. Dilute the solution to 250 ml. and determine the lactic acid by oxidation to CH_3CHO with dilute $KMnO_4$ in $MnSO_4$ according to Friedemann and Kendall (244, *cf.* Peters and Van Slyke, 516). Distill the acetaldehyde into sodium bisulfite and titrate the bound aldehyde with iodine.

Comment: Kendall and Friedemann (358) claim that 98 per cent of the alanine could be recovered by this method. They did not, however, test the procedure on protein hydrolysates.

McChesney (445) found that malic and citric acids would yield acetaldehyde and acetone respectively under the conditions of oxidation employed by Kendall and Friedemann (358) for the determination of lactic acid. He suggested that citric and malic acids

264

be removed by precipitation at neutrality with a *slight* excess of basic lead acetate. The lactic acid which remains in the filtrate is estimated by $KMnO_4$ oxidation.

McChesney (446) reported later that only 91 per cent of the alanine was converted into lactic acid under the conditions described by Kendall and Friedemann; while the production of volatile aldehydes, other than CH_3CHO, from the hydroxy acids of a protein hydrolysate, tended to yield high results.

Fürth *et al.* (253) removed the dicarboxylic amino acids from the hydrolysate by the Ritthausen-Foreman method (Chapter VI) and prevented the distillation of the higher volatile aldehydes by a good fractionating column. Recoveries of alanine added to protein hydrolysates varied from 91 to 96 per cent.

B. The Modifications of Fromageot and Heitz (245) and of Desnuelle (194)

Principle: Permanganate oxidation of the hydroxy acids prepared from a protein hydrolysate produces, in the presence of mercuric acetate, acetaldehyde only from lactic acid. The acetaldehyde is then estimated colorimetrically by means of the rather specific piperazine-sodium nitroprusside reaction.

Method: 1. Oxidation. Deaminate the amino acids from a protein hydrolysate by any convenient method (*cf.* A above, also Chapter V). To 10 ml. of a solution of the hydroxy acids, add 10 ml. of 2 N H_3PO_4, 10 ml. of 10 per cent $MnSO_4$, and 5 ml. of 5 per cent mercuric acetate in 1 per cent acetic acid. Then heat to boiling, and introduce 0.2 N $KMnO_4$ at the rate of 1 drop per second for 10 minutes. Heat 10 minutes longer. Trap the acetaldehyde in $NaHSO_3$.

2. Estimation of CH_3CHO. Destroy the excess $NaHSO_3$ with iodine and remove any surplus I_2 with a drop of 0.1 N $NaHSO_3$. Place 1 to 6 ml. of this solution in a test tube and dilute to 6 ml. with a solution containing all the reagents and in the same concentrations as they are present in the unknown except, of course, CH_3CHO. Add 1.5 ml. of 33 per cent aqueous piperazine and 0.5 ml. of a freshly prepared 4 per cent solution of sodium nitroprusside. Shake and read in a photoelectric colorimeter at the maximum deflection of the galvanometer within 2 minutes. Use light filter 560 or 570 mu. The color is proportional to the concentration of acetaldehyde over the range 200 to 700 gamma.

Comment: Recoveries of 94 to 97 per cent of alanine are reported by this method (245, 194). The use of the nitroprusside reaction eliminates the danger of high values due to the distillation of other volatile aldehydes.

The possible production of acetaldehyde from 1,2-dihydroxy-butyric acid, from threonine, does not appear to have been investigated.

C. The Procedure of Block, Bolling, and Webb (103, 104)

Principle: Alanine is deaminated to lactic acid which, in turn, is quantitatively oxidized to acetaldehyde by ceric sulfate according to the procedure of Gordon and Quastel (260). The CH_3CHO is estimated colorimetrically by Eegriwe's p-hydroxydiphenyl method (*cf.* Chapter IV).

Apparatus: Gas adsorption train (*cf.* Chapter IV, Section 1A, 1D).

Method: Hydrolyze 10 to 50 mg. of protein with a few ml. of 1:1 HCl under reflux for 16 to 20 hours. Evaporate off the excess HCl and dilute the residue with alcohol-free water. Deaminate the amino acids with 2 ml. of 13.8 per cent $NaNO_2$ and 2 ml. of 1:3 H_2SO_4 (*cf.* Chapter V). After standing 10 minutes at room temperature, destroy the excess HONO by warming the solution on the steam bath for the same length of time.

Dilute the solution of hydroxy acids so that 1 ml. contains the equivalent of 0.5 to 1.0 mg. of protein. Pipette a suitable aliquot of the solution into the oxidizing tube of the gas adsorption train used in the p-hydroxydiphenyl method for the microestimation of threonine (*cf.* Chapter V, Section 1D). Add 5 ml. of 10 per cent $Ce(HSO_4)_4$ in N H_2SO_4 and 10 ml. of water. The oxidation of lactic acid to acetaldehyde is complete in less than 30 minutes at 50°C., water bath. During this time the acetaldehyde is aerated into 15 ml. of concentrated sulfuric acid containing an excess of p-hydroxydiphenyl in the same way as described in Chapter IV, Section 1D, except that the stream of air carrying the aldehyde is dried by passing through a tube filled with soda lime. The remainder of the method is the same as that described for threonine.

Comment: 1,2-Dihydroxybutyric acid resulting from the deamination of threonine yields the theoretical quantity of acetaldehyde on oxidation with ceric sulfate. Therefore, in the presence of threonine, it is necessary to calculate the amount of alanine from the difference in total acetaldehyde formed by $Ce(HSO_4)_4$ oxidation after deamination and that quantity of CH_3CHO produced by $NaIO_4$ oxidation without deamination.

All precautions advised in the analogous threonine method should be taken.

Color formation can be enhanced by the addition of traces of cupric ion to the sulfuric acid (Barker and Summerson, 49).

Winnick's (684) use of the Conway (169, 170) micro-diffusion

technique may, also, be applicable to the estimation of alanine (*cf.* Chapter IV, Section 1C).

D. *Oxidation of Alanine to Acetaldehyde with Ninhydrin*
(Virtanen, Laine, and Toivonen, 663)

Principle: All amino acids except glycine yield NH_3, CO_2 and the next lower aldehyde on oxidation with triketohydrindenehydrate (ninhydrin) in weakly acid solution (Ruhemann, 559).

Method: 1. Hydrolysis and Precipitation of the Dicarboxylic Acids. Hydrolyze 1 gm. of protein with 10 ml. of concentrated HCl for 6 hours. Distill off the excess mineral acid and dilute the residue to 10 ml. Add 5 ml. of a 20 per cent suspension of $Ca(OH)_2$ and then introduce, with stirring, 100 ml. of ethanol. Remove the precipitate. Concentrate the filtrate to free it of ethanol and take up the residue in 50 ml. of water.

2. Oxidation. Place a suitable aliquot of the above solution containing 0.2 to 2.0 mg. of alanine, in a small round bottom flask with a ground glass joint and a side arm to be used as a gas inlet. Dilute the solution to 10 ml. and add 7.5 gm. of ammonium sulfate and 0.5 gm. of citric acid. Heat to a gentle boil and add 2 ml. of 1 per cent ninhydrin. Aerate for 30 minutes to carry the acetaldehyde formed into 4 ml. of 1 per cent $NaHSO_3$. Use a good fractionating column to prevent distillation of the higher aldehydes unless a specific method is employed for the determination of CH_3CHO.

3. Estimation. Remove the excess $NaHSO_3$ with 0.1 N iodine and then destroy the excess I_2 with the least quantity of 0.01 N $NaHSO_3$. Use starch as the internal indicator. Liberate the bound $NaHSO_3$ by the addition of an excess of $NaHCO_3$. Titrate the freed $NaHSO_3$ with 0.01 N I_2.

$$1 \text{ ml. of } 0.01 \text{ } I_2 \doteqdot 0.445 \text{ mg. of Alanine.}$$

Comment: The directions of Van Slyke *et al.* (636, 637, 638) and of Christensen *et al.* (161) for oxidation with ninhydrin may be used in place of those given above (*cf.* Chapter I, Part IV, Section 1).

Ninhydrin oxidation and the estimation of acetaldehyde by the more specific p-hydroxydiphenyl (206), (*cf.* Chapter IV, Section 1A) or piperazine-sodium nitroprusside (245, 194); (*cf.* this Chapter, Section B) methods appear to give promise of being the best microprocedures for the determination of alanine.

Neuberger and Sanger (474) found that aspartic acid did not yield appreciable quantities of acetaldehyde on oxidation with ninhydrin. They, therefore, did not find it necessary to carry out the initial Ritthausen-Foreman precipitation of the dicarboxylic amino acids.

PART III

ANALYTICAL VALUES

The amino acid values given in the following tables have been corrected to 16.0 per cent of nitrogen.

It should be kept in mind that the great majority of figures represent minimal or at best approximations to the true quantities of glycine and alanine present in the protein hydrolysate. However, certain general conclusions may be drawn from these data.

Animal Proteins: The connective, supporting, and elastic tissues are unusually rich in glycine, while milk, egg, and tissue proteins contain only small quantities of this amino acid. The large quantities of both glycine and alanine in silk fibroin are well known.

Glycine and Alanine in *Animal Proteins*

Calculated to 16.0 gm. N·

SOURCE	METHOD	REFERENCE		NITROGEN	GLYCINE	ALANINE
				per cent	gm.	gm.
Albuminoids:						
Gelatin	Bergmann	Bergmann	70	(18.3) ●	22.8	
Gelatin	Bergmann	Bergmann	70	(18.3)	23.6*	
Gelatin	Carbamate	Kingston	365		15.6	
Gelatin	Dakin-Fischer	Dakin	185	(18.0)	23	6
Gelatin	Kendall	Fürth	253	18.3		22
Gelatin	Town	Town	617	(16.0)	25.9	
Gelatin	Zimmermann	Patton	511	(16.0)	22.2	
Gelatin	Chromatographic	Gordon	261D			9.4
Collagen	Bergmann	Bergmann	70	18.6	22.8	
Collagen (Corium)	Zimmermann	Patton	511	(16.0)	6.9	
Elastin	Fischer	Abderhalden	4	(17.1)	24	6
Elastin	Bergmann	Stein	586	17.1	27.5*	4
Animals, Entire:						
Rat, Fetus	Zimmermann	Patton	511	(16.0)	10.6	
Chick, Normal	Zimmermann	Patton	512	(16.0)	9.3	
Chick, Pathological	Zimmermann	Patton	512	(16.0)	6.6ᵃ	
Blood Proteins:						
Fibrin	Isotope	Rittenberg	547	15.2	5.4*	
Hemoglobin-Horse	Fischer	Abderhalden	2	(16.7)		4
Hemoglobin-Horse	Zimmermann	Patton	511	(16.7)	<0.4	
Hemoglobins	Fromageot	Roche	552	(16.7)		7-8
Serum Globulin	Fischer	Abderhalden	5	(16.0)	5	2
Bence Jones	Fischer	Abderhalden	11	(16.0)	2	5
Egg Proteins:						
Albumin	Virtanen	Virtanen	663	12.1		7.4*
Albumin	Bergmann	Stein	588A	(15.5)	3.3	
Albumin	Zimmermann	Patton	511	(15.4)	1.9*	
Albumin	Fischer	Osborne	497	15.5	0	2
Vitellin	Fischer	Abderhalden	13	(16.3)	1	
Vitellin	Zimmermann	Patton	511	(16.3)	0.8	
Vitellin	Fischer	Osborne	495	16.3	0	<1

Glycine and Alanine in *Animal Proteins* (Continued)

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		NITROGEN	GLYCINE	ALANINE
				per cent	gm.	gm.
Livetin	Kendall	Jukes	348	15.5		6.4
Whole Egg	Zimmermann	Patton	512	16.0	2.2*	
Whole Egg	Zimmermann	Patton	511	(16.0)	2.7	
Keratins:						
Hair-Horse	Fischer	Abderhalden	9	(16.6)	4	1
Hair-Cow	Bergmann	Block	109	15.3	10.8	
Hair-Human	Bergmann	Block	97	15.4	4.5	
Wool	Bergmann	Block	97	15.4	6.8	
Wool	Fischer	Abderhalden	16	16.6	1	4
Wool	Chromatographic	Gordon	261B			4.0
Horn-Cattle	Fischer	Abderhalden	16	15.1	1	2
Horn-Cattle	Kendall	Fürth	253	18.3		2.2
Horn-Cattle	Bergmann	Block	97	16.1	9.7	
Feathers-Goose	Fischer	Abderhalden	10	(16.6)	2	2
Egg Membrane	Fischer	Abderhalden	14	(16.0)	4	4
Scyllium Stellare	Fischer	Pregl	529	15.1	3	3
Spongin	Fischer	Abderhalden	12	(16.0)	14	
Spongin	Fischer	Clancy	163		11	
Spongin	Bergmann	Block	96	13.0	17.7*	
Snake Skin	Bergmann	Block	97	15.2 ᵃ	13.8	
Silk Fibroin	Fischer	Abderhalden	20	19.0		21
Silk Fibroin	Bergmann	Bergmann	69	19.0	36.8*	22.2*
Silk Fibroin	Kendall	Fürth	253	(19.0)		18.4
Silk Fibroin	Fischer-Cherbuliez	Cherbuliez	156	(19.0)	29.8	
Silk Fibroin	Bergmann	Stein	588A	(19.0)	37.4	
Metallo Proteins:						
Ferritin	Zimmermann	Kuhn	396	8.4	2.1	
Muscle Globins	Fromageot	Roche	552	(16.0)		6–9
Hemocyanin	Fromageot	Roche	552	(16.0)		4–7
Milk Proteins:						
Casein	Fromageot	Desnuelle	194	15.7		2.8
Casein	Fischer	Foreman	240	15.6	<1	2
Casein	Kendall	Fürth	253	15.4		5.5
Casein	Fischer	Osborne	502	15.6	0	2
Casein	Zimmermann	Plimmer	523	14.0	0.6*	
Casein	Town	Town	617	(14.5)	4.5	
Casein	Virtanen	Virtanen	663	13.5		5.7*
Casein	Zimmermann	Patton	511	(15.4)	0.5	
Lactalbumin	Zimmermann	Patton	511	(15.5)	0.0	
Lactalbumin	Fischer	Jones	340	15.4	<1	
Muscle Proteins:						
Cod	Fischer	Abderhalden	24	13.6		7
Herring		Wakamatu	669	12.5	0	4
Chicken	Fischer	Osborne	493	(16.0)	<1	2
Scallop	Fischer	Osborne	496	17.1	0	
Beef	Fischer	Osborne	498	16.2	2	4
Myosin	Fischer	Sharp	575	16.8	2	4
Beef	Zimmermann	unpublished		16.1	5.0	
Tissue Proteins:						
Brain	Kendall	Kaplansky	354	15.2		5.8
Lens	Fischer	Hijikata	298	(16.0)		5
Liver	Zimmermann	unpublished		13.3	8.5	

* "Best Values."

ᵃ Chondrodystrophy.

Glycine and Alanine in *Plant Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE	NITROGEN	GLYCINE	ALANINE
			per cent	gm.	gm.
Autotropic Plants:					
Various	Bergmann, Brazier	Mazur 442		0.5–2.3	3.8–6.6
Biologically Active:					
Gramacidin	Kendall	Christensen 162	14.6		33
Gramicidin	Kendall	Hotchkiss 310	14.8		25–36
Gramicidin	Chromatographic	Gordon 261C		4.5–5.7	10.3
Tyrocidin	Kendall	Christensen 162	14.5		15
Tobacco Virus	Zimmermann, Bergmann	Ross 557	15.9	0.0	2.8
Yellow Enzyme	Fromageot	Desnuelle 194	16.3		8.1
Corn Proteins:					
Zein	Brazier	Brazier 129	17.5	0	5
Zein	Virtanen	Laine 398	(16.0)		9.9
Zein	Dakin-Fischer	Dakin 186	16.1		3
Zein	Kendall	Fürth 253	16.1		8.9
Zein	Fischer	Osborne 490	16.1	0	2
Zein	Fischer	Osborne 500	16.1		10
Zein	Virtanen	Virtanen 663	14.5		9.9*
Zein	•Zimmermann	Patton 511	(16.1)	0.0*	
Glutenin	Fischer	Osborne 490	(16.0)	<1	
Glutenin	Zimmermann	Patton 511	(16.0)	0.8	
Gluten	Zimmermann	unpublished	11.8	4.3	
Zein Residue	Zimmermann	unpublished	10.9	9.6	
Miscellaneous:					
Cottonseed Globulin	Fischer	Abderhalden 6	(18.6)	1	4
Cottonseed Meal	Zimmermann	unpublished	10.9	5.3	
Arachin	Fischer	Johns 322	18.3	0	4
Arachin	Zimmermann	Patton 511	(18.3)	1.8	
Peanut Meal	Zimmerman	unpublished	(10.4)	5.6	
Edestin	Fischer	Abderhalden 1	18.6	4	3
Edestin	Fromageot	Desnuelle 194	18.7		4.8
Edestin	Zimmermann	Patton 511	(18.7)	1.6*	
Glycinin	Zimmermann	Patton 511	(17.0)	1.4	
Hordein	Fischer	Kleinschmitt 369	17.2		1
Hordein	Zimmermann	Patton 511	(17.2)	0.0	
Sunflower Globulin	Fischer	Abderhalden 8	(18.6)	2	4
Wheat Proteins:					
Gliadin	Fischer	Abderhalden 7	(17.7)	1	3
Gliadin	Fischer	Osborne 489	17.7		2
Gliadin	Fischer	Osborne 503	17.7	0	<2
Gliadin	Zimmermann	Patton 511	(17.7)	<0.5	
Glutenin	Fischer	Osborne 489	17.5	<1	4
Glutenin	Zimmermann	Patton 511	(16.0)	0.8	
Gluten		Padoa 508	?	9	5
Gluten	Zimmermann	unpublished	13.5	7.2	

* "Best Values."

CHAPTER VIII

PROLINE AND HYDROXYPROLINE

	Proline	Hydroxyproline
Empirical Formula	C₅H₉O₂N	C₅H₉O₃N
Optical Form	*l*	*l*
Molecular Weight	115.08	131.08
Carbon	52.14	45.77
Hydrogen	7.88	6.92
Nitrogen	12.17	10.69
Oxygen	27.81	36.62
Melting Point	220–222°	270°

PART I

1. The Isolation of Proline and Hydroxyproline

A. *Fischer Ester Distillation (Fischer, 220, Van Slyke, 629)*

PRINCIPLE: The amino acid esters which distill below 90°C. and 0.5 mm. pressure are hydrolyzed and the solution of free amino acids is evaporated to dryness. The residue is then thoroughly extracted with absolute alcohol. Proline is determined in that portion of the residue which is completely soluble in cold absolute alcohol by precipitation as the copper salt, the cadmium chloride complex, the picrate or calculated from the nonamino nitrogen.

B. *Direct Solvent Extraction (Dakin, 183, 185)*

Principle: The excess mineral acid, H₂SO₄, is removed from the protein hydrolysate with baryta and the amino acid solution is adjusted with Ba(OH)₂ to the optimal reaction, usually about pH 6, for subsequent extraction. The amino acid solution is then extracted with normal butanol either at atmospheric pressure or preferably at a reduced pressure, until no appreciable quantities of amino acids are being extracted from the aqueous layer. The dried residue from the butyl alcohol solution is thoroughly extracted with absolute ethanol. Proline is determined in this ethanol solution by any of the procedures given in A above.

Under the proper conditions, hydroxyproline is not extracted by ethanol, but it can be removed from the mixed residue of monoamino acids by subsequent extraction with n-propyl alcohol. The residue from the propanol extraction is dissolved in a small volume

271

of water and all the amino acids except hydroxyproline are precipitated by the addition of 9 volumes of methanol. The hydroxyproline can be estimated in the 90 per cent methanol filtrate from the nonamino nitrogen.

C. *Dakin's Procedure According to Fürth and Minnibeck (252)*

Principle: After removal of the basic amino acids, proline and hydroxyproline are extracted with butanol (Dakin, 183, 185). The proline is separated from the other monoamino acids in the butyl alcohol extract by treatment with ethanol. The monoamino acid residue, free from proline, is then extracted with propyl alcohol to dissolve the hydroxyproline.

Method: Hydrolyze the protein with HCl and remove the excess HCl by concentration *in vacuo* and the NH_3 with lime. Precipitate the basic amino acids according to Van Slyke (*cf.* Chapter I) with phospho-24-tungstic acid. Remove the excess phosphotungstic acid from the filtrate with $Ba(OH)_2$ and the barium with H_2SO_4. Evaporate the amino acid solution to dryness in the presence of a diatomaceous earth. Extract the dried residue with hot butanol until no more material is removed.

Distill off the butyl alcohol and thoroughly extract the residue with absolute ethyl alcohol. Precipitate the proline from the alcoholic solution with $CdCl_2$. Calculate the proline from the nonamino nitrogen of the cadmium chloride precipitate. Correct for the solubility of proline cadmium chloride.

Extract the proline-free amino acid residue with n-propyl alcohol and after removal of the solvent, estimate the amount of hydroxyproline present from the nonamino nitrogen.

D. *The Precipitation of Proline and Hydroxyproline with Ammonium Reineckate (Kapfhammer and Eck, 353; Miller, 452)*

Principle: After removal of arginine, proline and hydroxyproline are precipitated from a weakly acid solution by an excess of ammonium reineckate, ammonium tetrarhanatodiamminochromate.

$$NH_4[Cr(NH_3)_2(SCN)_4] \cdot H_2O$$
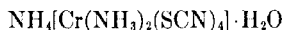
Reagents: Reinecke Salt (Dakin, 189). Heat 800 gm. (10.5 moles) of NH_4SCN at 145–150° to partly melt and add in 10 to 12 gm. portions with stirring, a mixture of 170 gm. (0.675 mol.) of powdered $(NH_4)_2Cr_2O_7$ and 200 gm. (2.6 mol.) of NH_4SCN. The temperature rises to 160° and a vigorous reaction takes place after adding 10 portions. Keep the temperature at 160° by adding the $(NH_4)_2Cr_2O_7$-NH_4SCN mixture. Continue stirring until the melt is

only warm, then add 750 ml. of ice water and filter after 15 minutes. Stir the precipitate with 2.5 liters of water at 65° and keep the suspension at 60° by warming. Filter the warm solution through a heated Buchner funnel. Crystallize out the ammonium reineckate by chilling the filtrate in the refrigerator over night. Warm the mother liquor to 60° to extract a second crop of reineckate from the residue of the initial aqueous extraction. Yield 50 to 57 per cent of theory.

Method: 1. Removal of Arginine. Adjust the decolorized protein hydrolysate to weakly acid to Congo red paper and precipitate the arginine as the diflavianate by the addition, at room temperature, of 3 to 4 moles of aqueous flavianic acid (*cf.* Chapter I).

2. Precipitation of Proline and Hydroxyproline. Warm the arginine diflavianate filtrate to 60° and add an excess of a warm 8 per cent solution of ammonium reineckate. Stand at 4°C. over night. Remove the precipitate and concentrate the filtrate *in vacuo* to less than one half of the initial volume. Add more reineckate at 60° and let stand in the cold to obtain a second crop of proline and hydroxyproline reineckates. Wash the precipitates with ice water and suck dry on the filter.

3. Decomposition of Reineckates. Suspend the washed precipitates in 50 per cent methanol and add an excess of $CuSO_4$. Remove and wash the precipitate. Remove the excess chromium ion with a stream of SO_2, the CNS^- ion with Ag_2SO_4, and the excess Ag^+ and Cu^+ with H_2S. Make the solution alkaline with barium hydroxide, remove the NH_3 by distillation *in vacuo* and the Ba^{++} with the needed quantity of H_2SO_4.

4. Separation of Proline and Hydroxyproline. Evaporate the amino acid solution to dryness and extract the residue with absolute alcohol. Proline is soluble in cold alcohol, hydroxyproline is not. The quantities of each acid can be estimated from the monoamino nitrogen in the alcoholic solution (proline) and in the residue (hydroxyproline).

The solubility of proline in absolute ethanol is 1.55 gm. per 100 gm. at 19°.

Proline reineckate $C_5H_9O_2N \cdot C_4H_7N_6S_4Cr$

Hydroxyproline reineckate $C_5H_9O_3N \cdot C_4H_7N_6S_4Cr \cdot 3H_2O$

Comment: Kapfhammer and Eck (353) point out that the reineckate method is not quantitative. They were able to recover only 84 per cent of proline and 79 per cent of hydroxyproline.

Dakin and West (188) have greatly simplified the decomposition of reineckates. Their procedure, which should be applicable to the

decomposition of proline and hydroxyproline reineckates, is as follows: The washed air dried precipitate of reinecke salts is shaken with the minimal quantity of methanol until the precipitate has dissolved. The solution is then heated to 50° in a water bath and an excess of dimethylaniline is added. The mixture is shaken on the machine for 30 minutes and then diluted with one half its volume of water. The bulk of the reinecke salt is precipitated as the dimethylaniline reineckate. The methanol is then removed by concentration *in vacuo* and the excess dimethylaniline and remaining reinecke acid is removed by repeated extraction, 4 or 5 times, with amyl alcohol.

E. Isolation of Proline by Means of Its Copper Salt
(Town, 616; Brazier, 129)

Principle: After removal of the sulfuric acid used for hydrolysis, (366), the amino acids are converted to their copper salts by boiling with $CuCO_3$. The copper salts are evaporated to a thick syrup and then thoroughly dried by repeated extraction with acetone. The powdered copper salts are then extracted 6 times with absolute methanol on the machine. The alcoholic solutions are separated and the copper is removed with H_2S. The amino acid solution is evaporated to dryness and the proline is extracted from the residue with absolute alcohol.

F. Isolation of Proline as the Betaine (Engeland, 210, 211, 212, 53)

Principle: The amino acids in a protein hydrolysate are exhaustively methylated with CH_3I or $(CH_3)_2SO_4$ and 10 per cent KOH. The stachydrine is first precipitated with $HgCl_2$, the precipitate is decomposed, and the betaine residue is fractionated with absolute alcohol. Stachydrine is isolated from the alcoholic solution as the platinic or auric chloride.
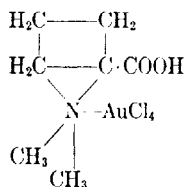
G. Isolation of Proline Rhodanilate (Bergmann, 65, 70, 587)

Principle: Proline forms relatively insoluble salts with rhodanilic acid, tetrathiocyanato-dianilidochromiato acid, $H[Cr(CNS)_4 (C_6H_5 \cdot NH_2)_2]$ (*cf.* the formula for reinecke acid).

Reagents: Aniline Rhodanilate. Heat 500 gm. of chrome alum, 600 gm. of KCNS, and 500 ml. of water on the steam bath for 4 hours. Cool and add 500 ml. of aniline, then keep at 60° for 3 hours. Cool and pour the solution into a mixture of 6000 ml. of water and 600 ml. of acetic acid. After standing for some hours, remove the precipitate and extract the residue with 1.5 to 2 liters of cold methanol. Filter the methanol and precipitate the salt by pouring the alcoholic solution into 6 liters of water. Stir constantly. Remove the precipitate and purify it by dissolving in methanol and reprecipitating with water.

Yield 330 gm. Nitrogen 17.1 per cent (theory).

Ammonium Rhodanilate. Dissolve 400 gm. of the aniline salt in a mixture of 600 ml. of methanol and 300 ml. of concentrated ammonia. Cool in ice. Then slowly add 3 liters of water to the ice cold solution. Purify the precipitate by repeating the above procedure.

Then dissolve the ammonium salt in cold methanol, add charcoal, and slowly pour the filtered solution into an excess of ice water. Wash the precipitate with ice water and repeat the charcoal treatment. Dry the residue in the dark at 0° on a porous plate. Keep the salt in the cold.

Method: 1. Purification of Hydrolysate. Remove the humin from the hydrolysate by the *in situ* formation of copper sulfide from $Cu(OH)_2$ and H_2S.

2. Precipitation of Proline. Use the equivalent of 2.48 gm. of gelatin for each precipitation. Add 275 ml. aliquots of the clarified gelatin hydrolysate, in dilute HCl, to 1.695, 2.001, and 2.301 gm. of purified ammonium rhodanilate in 175 ml. portions of cold methanol. Keep the mixtures at 0° for 16 hours, then shake on the machine at 0° for 4 hours, and again keep at 0° for 20 hours. Filter the proline rhodanilate precipitates at 0°. Do not wash.

3. Estimation of Proline. Decompose the precipitates with dilute acetic acid and dimethylaniline. Calculate the quantity of proline present in each precipitate by the optical rotation. Then calculate the amount of proline in the protein by the solubility product from the formula

$$(R^1 - X_a)(Y - y_a) = (R^2 - X_b)(Y - y_b)$$

where R^1, R^2, are the moles of ammonium rhodanilate used.

X_a, X_b are the moles of ammonium rhodanilate precipitated.

y_a, y_b are the moles of proline rhodanilate precipitated.

Y is the molar quantity of proline in x ml. of the hydrolysate.

Comment: Bergmann's (65) original method for the determina-

tion of proline by precipitation with rhodanilic acid could not be confirmed by numerous competent investigators. The general procedure described above appears to be rather satisfactory. However, the published accounts give values for only a few proteins.

Ing and Bergmann (315) have described a micromodification of the rhodanilate method which includes a description of the apparatus used (cf. Chapter IX, Section 10).

The optimal conditions have to be worked out for each protein hydrolysate.

2. THE COLORIMETRIC ESTIMATION OF PROLINE AND HYDROXYPROLINE

A. The Estimation of Hydroxyproline (Lang, 400; Waldschmidt-Leitz, 671)

Principle: Hydroxyproline can be oxidized and decarboxylated to pyrrol by NaOCl. The pyrrol is then condensed with isatin or p-dimethylaminobenzaldehyde to yield a colored compound.
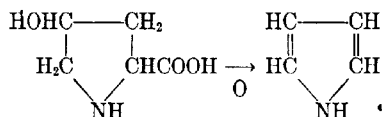
Reagents: Sodium Hypochlorite (Raschig): pass Cl₂ gas into a mixture of 80 gm. of NaOH, 160 ml. of H₂O, and 600 gm. of ice until the alkali has taken up 71 gm. of chlorine. Dilute the solution to 1 liter. Keep cold.

p-Dimethylaminobenzaldehyde: dissolve 2.6 gm. in 100 ml. of ethanol.

Isatin: dissolve 100 mg. in 200 ml. of concentrated H_2SO_4.

Pyrrol Standard: dissolve 100 mg. of pyrrol in 1 liter of N/200 NaOH. Dilute 1:10 before use.

Method: 1. Oxidation. To an ice cold solution containing 0.2 to 3.0 mg. of hydroxyproline, add 0.5 ml. of 10 per cent Na_2CO_3 and 0.5 ml. of NaOCl for each 10 ml. of amino acid solution. Wait 2 to 3 minutes and then add 1 ml. of 11.6 per cent sodium glutamate. Steam distill the pyrrol until 100 ml. of distillate comes over.

2. Color Development. A. With p-Dimethylaminobenzaldehyde. Dilute an aliquot of the distillate containing 0.01 to 0.1 mg. of pyrrol to 20 ml. and add 1 ml. of dimethylaminobenzaldehyde solution and 1 ml. of 6 N HCl. Allow the color to develop at room temperature (20 to 30°) for 15 minutes. Read using filter 570 mu.

B. With Isatin. Add 2 ml. of a saturated aqueous solution of

$HgCl_2$ to 20 ml. of the distillate containing 0.05 to 0.3 mg. of pyrrol. Centrifuge and wash the precipitate of pyrrol mercuric chloride with two 5 ml. portions of ethyl alcohol at 60°. Cool before centrifuging between each washing. Dissolve the mercury precipitate in 2 ml. of water, a few drops of 5 per cent NaCl, and 1 ml. of 6 N HCl. Dilute the solution to either 10 or 25 ml. To a small aliquot add 1 ml. of isatin-sulfuric acid solution. Heat the mixture in a boiling water bath for 10 minutes and read the color in a colorimeter using filter 570 mu.

Comment: Lang (400) originally believed that the color formed with dimethylaminobenzaldehyde gave the sum of proline plus hydroxyproline and that formed with isatin was specific for hydroxyproline. Waldschmidt-Leitz and Akabori (671) showed that hydroxyproline alone yielded pyrrol after oxidation with NaOCl. They believed that Lang's results were due to the presence of some hydroxyproline in his proline standards.

Waldschmidt-Leitz and Akabori (671) found the average yield of pyrrol from hydroxyproline to be approximately 80 per cent and they, therefore, correct by the factor 1.25. However, the yield of pyrrol is highly dependent on the conditions employed and especially on the quantity of NaOCl used. It is the authors' opinion that the procedure requires further study.

Kuhn and Desnuelle (393) estimate the quantity of pyrrol present in 1 ml. of distillate by adding 1 ml. of 1.19 sp. gr. HCl and 1 ml. of 2.5 per cent alcoholic p-dimethylaminobenzaldehyde. The condensation is allowed to take place at 37° for 20 minutes. The solution is cooled and the volume is brought to 20 ml. The color is read using filter 530 mu.

B. The Oxidation of Hydroxyproline with Hydrogen Peroxide
(McFarlane and Guest, 449)

Principle: Hydroxyproline is oxidized with H_2O_2 to yield pyrrol which then is determined by condensing with isatin.

Method: 1. Oxidation. To 1 ml. of a neutralized protein hydrolysate, containing 0.4 to 1.6 mg. of hydroxyproline, add 1 ml. of 0.01 M $CuSO_4$, 1 ml. of 10 per cent NaOH, and 1 ml. of 6 per cent H_2O_2. Shake for 5 minutes, then place in a boiling water bath for 5 minutes. Cool and add 1.5 ml. of 2 N HCl. Dilute the solution to 10 ml.

2. Development of Color. To 1 ml. of the pyrrol solution, add 1 ml. of freshly prepared 0.01 per cent isatin in water and 1 ml. of 2 N HCl. Place the mixture in a boiling water bath for exactly 3 minutes. Stand for 5 minutes and then cool in water. Dilute the

solution to 10 ml. and read the color using filter 520 mu against a
blank prepared in the same way except that the solution is not
heated after the addition of isatin.

Comment: Guest, and McFarlane (268) found that 96 to 97 per
cent of the pyrrol in 5 ml. of solution (0.8 mg. of pyrrol) was pre-
cipitated by the addition of 5 ml. of saturated aqueous $HgCl_2$ in
the presence of 5 ml. of pH 6.3 phosphate buffer. They also showed
that 0.005 to 0.030 mg. of pyrrol could be estimated in 1 ml. of
solution by adding 0.2 ml. of 0.05 per cent isatin in glacial acetic
acid and 1 ml. of concentrated HCl. After standing 5 minutes, the
colored solution was diluted to 10 ml. with ethanol and read in a
colorimeter using light filter 660 mu.

C. *The Colorimetric Estimation of Proline (Guest, 269)*

Principle: Proline is oxidized with lead peroxide to yield pyrrol
(?) which, in turn, is estimated by condensing it with p-dimethyl-
aminobenzaldehyde.

Method: 1. Oxidation. To 5 ml. of a protein hydrolysate, contain-
ing 2 to 10 mg. of proline, add N NaOH to pH 8.7. Then add 10 ml.
of M phosphate buffer of pH 8.7 and 1 gm. of PbO_2. Boil the suspen-
sion under reflux for 30 minutes. Cool, filter, and dilute the filtrate
to 200 ml.

2. Estimation. Add 5 ml. of water, 1 ml. of 5 per cent p-dimethyl-
aminobenzaldehyde in ethanol and 1 ml. of 2 N HCl to 5 ml. of the
oxidized filtrate. Heat the solution in boiling water for 1 minute.
Then after 5 minutes, cool the colored solution in water. Read using
filter 520 mu.

Comment: Hydroxyproline gives approximately the same amount
of color under these conditions. The procedure, therefore, gives an
approximate indication of the sum of proline and hydroxyproline
present.

CHAPTER VIII

PART II

ANALYTICAL RESULTS

The analytical figures given in the following tables have, as previously, been recalculated to 16.0 per cent of nitrogen. In most cases, the values have little absolute quantitative significance, but can be used only as rough indications of the existence of these two amino acids in a protein hydrolysate.

The relatively large amounts of the prolines in gelatin, zein, and gliadin, all of which are nutritionally inadequate proteins, are noteworthy.

Proline and Hydroxyproline in *Animal Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		PROLINE	HYDROXY-PROLINE
				gm.	gm.
Albuminoids:					
Gelatin	Engeland	Bastian	53	24–25	
Gelatin	Bergmann	Bergmann	65	18	<13
Gelatin	Bergmann	Bergmann	70	15.4	
Gelatin	Dakin	Dakin	185	8.5	<13
Gelatin	Dakin-Fürth	Fürth	252	7.9	12.9
Gelatin		Gordon	261B	15.0	
Gelatin		Kingston	365	19.5	18.6
Gelatin	Lang	Lang	400	9	13
Gelatin	McFarlane	McFarlane	449		13.0–13.5*
Gelatin	Bergmann	Stein	587	15.3±0.4*	
Gelatin	Lang	Waldschmidt	671		9.4
Collagen	Bergmann	Bergmann	70	15.0	
Elastin	Engeland	Engeland	211	4	
Elastin	Bergmann, Kapfhammer	Stein	586	14.2	1.9
Biologically Active Subs.:					
Secretin		Ågren	26	5	
Blood Proteins:					
Hemoglobin	Bergmann	Bergmann	67	2	
Globin	Lang	Lang	400		0
Seralbumin	Lang	Lang	400		0
Serglobulin	Lang	Lang	400		0
Bence Jones	Fischer	Abderhalden	11	2	
Bence Jones	Bergmann	Devine	195	6.9	0
Bence Jones	Fischer	Hopkins	308	3	
Egg Proteins:					
Albumin	Dakin	Calvery	139	4.3	
Albumin	Fischer	Osborne	497	4	
Vitellin	Fischer	Abderhalden	13	3	
Livetin	Kapfhammer	Jukes	348	2–3	

279

Proline and Hydroxyproline in *Animal Proteins* (Continued)

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		PROLINE	HYDROXY-PROLINE
				gm.	gm.
Keratins:					
Hair, Horse	Fischer	Abderhalden	9	4	
Hair, Cow	Bergmann	Block	109	8.5	
Wool		Martin	437	6.4	
Wool	Fischer	Abderhalden	16	4	
Wool		Gordon	261B	9.3–6.8	
Feathers	Fischer	Abderhalden	9	4	
Horn	Fischer	Abderhalden	16	4	
Horn	Fischer	Fischer	224	4	
Egg Membrane	Fischer	Abderhalden	14	4	
Egg Membrane	Dakin	Calvery	142	3.7	
Spongin	Fischer	Abderhalden	12	6	
Spongin	Fischer	Clancy	163	6	
Scyllium stellare	Fischer	Pregl	529	5	
Silk Fibroin	Fischer	Abderhalden	20	1	
Milk Proteins:					
Casein	Dakin-Van Slyke	Dakin	183	7–9	
Casein	Engeland	Engeland	210	7	
Casein	Fischer-Van Slyke	Foreman	240	7.8	
Casein	Dakin-Fürth	Fürth	252	5.9	2.2
Casein	Guest	Guest	269	8.2	
Casein	McFarlane	McFarlane	449		0
Casein	Fischer	Osborne	502	5	
Casein	Fischer-Van Slyke	Van Slyke	629	7.0	
Casein	Lang	Waldschmidt	671		0
Lactalbumin	Fischer	Jones	340	4	
Miscellaneous Proteins:					
Protamine	Brazier	Hirokata	299	<10	
Protamine	Lang	Waldschmidt	671		0
Kidney	Lang	Lang	400		0
Muscle Proteins:					
Cod	Kapfhammer	Abderhalden	24	3	1
Fish	Fischer	Osborne	493	3	
Herring		Wakamutu	669	>3	
Animal	Lang	Lang	400		0
Chicken	Fischer	Osborne	493	5	
Ox	Fischer	Osborne	498	6	
Myosin	Fischer	Sharp	575	<1	

Proline and Hydroxyproline in *Plant Proteins*

Calculated to 16.0 gm. N.

SOURCE	METHOD	REFERENCE		PROLINE	HYDROXY-PROLINE
				gm.	gm.
Autotropic Organisms:					
Marine Algae	Bergmann, Kapfhammer	Mazur	442	9–10	2–6
Biologically Active Proteins:					
Gramicidin	Bergmann	Christensen	162	0	0
Tyrocidine	Bergmann	Christensen	162	0	0
Tyrocidine	Chromatographic	Gordon	261E	7.7–8.5	
Tobacco Virus	Bergmann	Ross	556	4.7	
Tobacco Virus	Bergmann	Ross	557	5.5	
Ricin	Fischer	Karrer	355	4	0
Yellow Enzyme	Van Slyke, Lang	Kuhn	393	6.4	0
Corn Proteins:					
Zein	Brazier	Brazier	129	8.9	
Zein	Dakin-Van Slyke	Dakin	186	9–12	0
Zein	Dakin-Van Slyke	Fürth	252	8.4	0.8
Zein	Fischer	Osborne	490	7	
Zein	Fischer	Osborne	499	9	
Glutenin	Fischer	Osborne	490	5	
Miscellaneous Proteins:					
Arachin	Fischer	Johns	322	<2	
Cottonseed Globulin	Fischer	Abderhalden	1	2	
Coconut Globulin	Dakin	Johns	334	5	
Edestin	Fischer	Abderhalden	1	2	
Edestin		Gordon	261	5.4	
Edestin	Fischer	Osborne	500	4	
Grass	Kapfhammer	Miller	452	2.5	
Hordein	Fischer	Kleinschmitt	369	6	
Lupin Meal		Heinrich	286	4	
Mold	Brazier	Woolley	689	<2	
Soybean Meal		Heinrich	286	5	
Sunflower Seed Globulin	Fischer	Abderhalden	8	3	
Wheat Proteins:					
Gliadin	Fischer	Abderhalden	7	2	
Gliadin	Fischer	Osborne	489	7	
Gliadin	Fischer	Osborne	503	12	
Gluten	Engeland	Engeland	211	10	
Gluten		Padoa	508	8	
Glutenin	Fischer	Osborne	489	4	

GENERAL METHODS

PART I

HYDROLYSIS AND PREPARATION OF THE SAMPLE FOR ANALYSIS

HYDROLYSIS

IT IS recognized that one of the most serious difficulties in protein analysis is the initial hydrolysis of the protein to amino acids. With the exception of the spectrographic methods for the aromatic amino acids (*cf.* Chapter II) and certain tests for cystine (*cf.* Chapter III), the only general procedure which gives promise of accuracy, without preliminary hydrolysis, is the important observation of Beadle and Tatum (60) that single ascospore strains of neurospora can be produced by x-rays which will grow normally on a complete medium, but scarcely at all on a medium devoid of one ingredient. Thus from approximately 2,000 strains so produced, three mutants have been found. One of these is unable to synthesize pyridoxine, another thiamine, and the third is unable to grow without added p-amino-benzoic acid. If analogous strains, from this or other organisms, can be developed for amino acids in the *intact protein molecule*, a whole new and infinitely improved method will be opened up.

Proteolytic enzymes are the most active catalytic agents known at present, but suffer from several disadvantages. (a) The hydrolysis seldom goes to completion. (b) Due to the heat lability of the proteolytic enzymes, the temperature of the reaction usually has to be maintained at 80°C or less. (c) The enzymes are proteins and often undergo partial autolysis with the result that a portion of the amino acid which is to be estimated may have arisen from the enzyme itself.

Strongly dissociated acids and alkalies are, therefore, usually employed. The concentration of the reagent to be used in any specific case is somewhat dependent on the protein to be hydrolyzed, but *is inversely proportional to the time and temperature of the reaction*. It is usual to employ a volume of reagent equal to 5 to 20 times the weight of the protein to be hydrolyzed. When the hydrolysis is carried out at atmospheric pressure, the following concentrations are usually employed:

HCl	3 to 12 N, commonly 6 N
H₂SO₄	4 to 8 N, commonly 8 N
HI	commonly 57 per cent
NaOH	commonly 5 N
Ba(OH)₂	commonly 14 per cent

When the temperature of the reaction is raised, the time of hydrolysis or the concentration of the reagent can be proportionally reduced. Thus at 20 lbs. pressure, 20 volumes of 4 per cent H_2SO_4 for 10 hours will suffice where 10 volumes of 40 per cent H_2SO_4 for 20 hours were required at atmospheric pressure. Gilson, *et al.* (257) have found that 100 gm. of casein could be hydrolyzed by 150 gm. of oxalic acid and 300 ml. of water at 15 lbs. pressure for 40 hours.

Steinhardt and Fugitt (589) have reported that certain organic acids such as cetylsulfonic, dodecylsulfonic, dodecylsulfuric, n-tetradecylsulfate half ester, etc., are more active catalysts for the hydrolysis of amide and peptide bonds in proteins than the mineral acids commonly employed. This interesting development has not, to the authors' knowledge, been used as yet by protein analysts. They (589) used approximately 100 gm. of 0.02 to 0.15 M acid per gm. of protein. The temperature of their experiments varied from 65 to 75°.

It is usually necessary to remove the excess of the reagent used in the hydrolysis. Sulfuric acid is commonly precipitated by baryta or lime, hydrochloric acid is removed by distillation *in vacuo* followed by precipitation with silver oxide or preferably with cuprous oxide, hydriodic acid is removed with silver oxide or silver chloride, while barium hydroxide is precipitated with sulfuric acid or CO_2. Block (107) has found that the excess mineral acids can be removed by synthetic anion exchange resins (Deacidite, Amberlite IR-4) without any loss of amino acids.

ESTIMATION OF THE COMPLETENESS OF HYDROLYSIS

Principle: The liberation of carboxyl and amino groups from peptide linkage is the object of protein hydrolysis. Hydrolysis is considered complete when a maximum number of –COOH or –NH₂ groups have been liberated. The carboxyl groups can be readily estimated by one of the modifications of the Schiff-Sørensen formol titration method (*cf.* Schmidt, 569, pp. 189 to 198) while the amino nitrogen can be determined by the Van Slyke method (*cf.* Schmidt, 569, pp. 198–203).

A. *The Amino Nitrogen Titration Method of Pope and Stevens (525)*

Principle: Amino acids form soluble copper salts. The copper content and consequently the NH_2 N of the filtrate is determined iodometrically.

Reagents: Cupric Chloride: 27.3 gm. per liter (0.16 M).

Trisodium Phosphate: Dissolve 64.5 gm. of Na_2HPO_4 in 500 ml. of CO_2-free water. Add 7.2 gm. of NaOH and dilute the solution to 1000 ml.

Borate buffer: Dissolve 57.21 gm. of sodium borate in 1500 ml. of water, add 100 ml. of N HCl and dilute to 2 liters.

Cupric Phosphate suspension: Mix 1 volume of $CuCl_2$ and 2 volumes of Na_3PO_4, then add 2 volumes of borate solution. Prepare fresh every few days.

Thymolphthalein: 250 mg. in 100 ml. of 50 per cent ethanol.

Sodium Thiosulfate: Dissolve 49.6 gm. of $Na_2S_2O_3$ in 200 ml. of CO_2-free water and dilute to 2000 ml., add 0.1 per cent borate buffer and dilute to 0.01 N before use with water containing a little borate buffer.

Potassium Iodate: Dissolve 356.75 mg. of dried KIO_3 in 1 liter of H_2O.

Starch: Prepare a 1.0 per cent solution of starch. Adjust the solution to pH 7.

Method: To 1 to 5 ml. of amino acid solution, add 4 drops of indicator and N NaOH to a faint blue color. Then add 30 ml. of the $Cu_3(PO_4)_2$ suspension with mixing. Dilute to 50 ml. and filter.

To 10 ml. of the filtrate add 0.5 ml. of acetic acid and 2 gm. of KI. Titrate the I_2 with 0.01 N $Na_2S_2O_3$ using starch as the internal indicator.

$$1 \text{ ml. of } 0.01 \text{ N } Na_2S_2O_3 \approx 0.28 \text{ mg. of Amino N}$$

PREPARATION OF SAMPLE FOR ANALYSIS

Although methods for the preparation of the protein sample for analysis have been mentioned briefly earlier in this monograph, the general principles are obviously simple. The protein must be prepared as devoid as possible from contaminating substances, especially carbohydrates and fats.

The removal of fatty substances usually offers no great difficulty as successive extraction with acetone, hot alcohol, hot benzene, and anhydrous ether will almost always reduce the residual lipoids to very small amounts.

On the other hand, the complete removal of carbohydrates and related compounds, especially from plant proteins, without chang-

ing the composition of proteins to be analyzed is usually difficult and often impossible. When, for example, the entire protein fraction of the cereal grains is to be analyzed, a considerable part of the carbohydrate moiety can be removed with the aid of amylolytic enzymes or the proteins can be solubilized, and so separated from the carbohydrates by means of hot dilute mineral acids (4 per cent HCl) or by the use of proteolytic enzymes.

Heat coagulation is often employed to separate soluble proteins from inorganic salts, etc. It is usually assumed that the amino acid composition of the heat coagulated protein is the same as that of the original substance. However, Calvery, Herriot, and Northrup (146) have shown that the amino acid composition of pepsin and of the heat coagulum obtained from it are not identical. Kiesel and Kusmin (363) have reported similar results with heat coagulated edestin and the undenatured protein. The importance of these experiments is obvious.

Chibnall, Rees, and Williams (157A) point out that certain proteins are so hygroscopic when anhydrous that they cannot be handled without special precautions; moisture and nitrogen contents should therefore be determined on separate samples of the air dried protein.

DETERMINATION OF NITROGEN

Chibnall, Rees, and Williams (157A) in confirmation of the earlier investigators (Osborne, Sørensen, etc.) have again proved that proteins and protein hydrolysates, especially those rich in lysine and histidine must be digested 8 hours or more after the Kjeldahl liquid is clear even when catalysts and micro quantities are used. This is seldom done.

SEPARATION OF AMINO ACIDS

1. The Fractional Distillation of Amino Acid Esters (Fischer, 219)

Principle: Emil Fischer (219) showed in 1901 that the ethyl esters of certain amino acids could be separated by fractional distillation *in vacuo*. This procedure was based on the observation of Curtius in 1883 that glycine ester could be distilled unchanged.

Method: Details of this method which are readily available in "The Biochemistry of the Amino Acids" by Mitchell and Hamilton (462), in "The Chemistry of the Amino Acids and Proteins" by Schmidt (569, pp. 138 to 146) and in other standard text and reference books on proteins, will not be repeated here.

The method of esterifying the amino acids employed by Osborne and his collaborators was based on the experiments of Phelps and Phelps (517) who distilled the organic acids with absolute alcohol containing 1.25 per cent HCl and 1 per cent $ZnCl_2$ while passing in a stream of gaseous absolute alcohol. The rates of distillation and of instillation of alcohol were adjusted so as to keep the volume in the esterifying flask constant. The esterification is repeated two or three times.

To reduce the hydrolysis of the esters during the removal of the HCl with alkali, Foreman (240) used the following procedure based upon the finding of Zelinsky *et al.* (696). After removal of the dicarboxylic amino acids with $Ca(OH)_2$ and ethanol, the lead salts of the amino acids are prepared by heating the hydrolysate with an excess of litharge. The lead salts are dried and suspended in absolute alcohol and the suspension is saturated with HCl gas. The precipitate of $PbCl_2$ is removed and washed with ethanol and the alcoholic solution is neutralized to about pH 6 with ammoniacal absolute alcohol. The precipitate of NH_4Cl is removed and washed with ethanol and the ethanol is distilled off. The residue is taken up in dry $CHCl_3$ and any precipitate of NH_4Cl is filtered off. The bound HCl is removed from the esters by dry $Ba(OH)_2$ at low temperature. The $BaCl_2$ is filtered and washed with $CHCl_3$ and after removal of the chloroform, the amino acid esters are distilled *in vacuo*.

Cherbuliez *et al.* (156) acetylated the amino acid esters with an

excess of acetic anhydride in the presence of sodium acetate before fractional distillation, while Gurin (273) distilled the N acyl amino acid esters in a high vacuum, 10^{-6} to 10^{-7} mm. The amino acids were acylated with benzene sulfonyl chloride in alkaline solution.

Comment: Osborne and Jones (501) In 1910 made the following comments. "The high hopes raised by the analytical methods introduced by Emil Fischer appear to have led to the assumption that we should soon know practically all of the constituents of the more important proteins . . . : It is plain . . . that there is a widespread feeling of disappointment." Their experiments with mixtures of the pure amino acids resulted in recoveries of from 0 in the case of serine, to 88 per cent in the case of leucine, with a mean recovery of about 60 per cent. The failure to recover serine may be explained by the finding of Jones and Johns (322) that serine ester is not extracted by ether in the presence of $BaCl_2$ and $Ba(OH)_2$.

2. Exhaustive Methylation of Amino Acids (Engeland, 210, 211)

Method: This procedure, which has been used only for the estimation of proline and aspartic acid, has been described in the Chapters devoted to these amino acids.

3. The Precipitation of Amino Acids as the Mercury Carbamates (Neuberg and Kerb, 473)

Principle: All amino acids, except proline and valine, form insoluble salts with mercuric acetate in the presence of Na_2CO_3 and alcohol.

$$\begin{array}{ccc} \underset{\underset{NH_2}{|}}{RCHCOOH} & +Na_2CO_3 \rightarrow & \underset{\underset{NHCOONa}{|}}{RCH \cdot COONa} & +Hg(COOCH_3)_2 \end{array}$$

$$\rightarrow \underset{\substack{| \\ NH—COO}}{RCH—COO} \Big\rangle Hg \quad \xrightarrow[Hg(OOCCH_3)_2]{Na_2CO_3} \quad \underset{\substack{| \\ NH—COO}}{RCH—COO} \Big\rangle Hg \cdot HgO$$

white precipitate   red-yellow precipitate
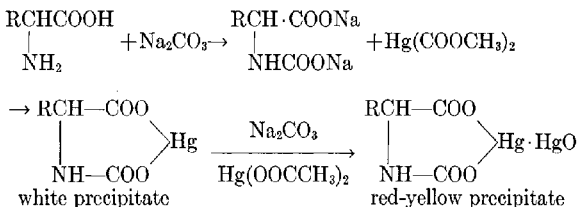
Method: Add a 25 per cent solution of mercuric acetate in water or ethanol containing a few drops of acetic acid and a 10 per cent aqueous solution of Na_2CO_3 alternately with stirring to a solution of amino acids until no further white precipitate forms. Then add a slight excess of the reagents until the precipitate becomes a yellowish red. Precipitate the mercury salts with 5 to 8 volumes of alcohol. Wash the precipitate with 80 per cent ethanol and liberate the amino acids with H_2S as usual.

Comment: Woolley and Peterson (689) found that the Neuberg-Kerb reagent did not precipitate all the amino acids and that there is a further loss due to adsorption (?) on the HgS precipitate.

4. Oxidation of Amino Acids with Ninhydrin (Ruhemann, 559)

Principle: All naturally occurring α-amino acids, except glycine, are deaminated and decarboxylated to give NH_3, CO_2 and the next lower aldehyde when warmed in dilute acid with ninhydrin, triketohydindene hydrate (Ruhemann, 559). This reaction has been used by Van Slyke *et al.* (636, 637, 638) for the estimation of lysine in phosphotungstic acid precipitates (*cf.* Chapter I); for glutamic and aspartic acids in mixtures (*cf.* Chapter VI); and by Virtanen, *et al.* (662, 663) for determination of alanine (*cf.* Chapter VII).

5. Extraction of Monoamino Monocarboxylic Acids with Butanol (Dakin, 183, 185)

Principle: Dakin (183, 185) found that the monoamino mono-carboxylic acids, at approximately neutral *p*H, could be extracted from a concentrated aqueous solution with warm butyl and iso-propyl alcohols. The details of this procedure are given in Schmidt (569, pp. 142–146; *cf.* 506).

Comments: This procedure, which appeared to have considerable promise, has not proved satisfactory as a method for the quantitative separation of the mono from the dibasic and dicarboxylic amino acids. Johns and Jones (334) found alanine in the aqueous residue and glutamic acid in the butanol extract. Other investigators have had similar difficulties.

A useful extraction apparatus has been described by Woolley (690).

6. Fractionation of the Copper Salts (Ehrlich, 207; Brazier, 129)

Principle: The copper salts of the amino acids are prepared by boiling a protein hydrolysate with an excess of $Cu(OH)_2$ or $CuCO_3$. The solution is evaporated to a syrup and the salts are thoroughly dried by the aid of acetone. Three groups of copper salts are obtained by successive extraction with different solvents.

A. Valine, hydroxyvaline (?), proline, isoleucine, some leucine and tyrosine are soluble in dry methanol.

B. Alanine, tyrosine, glutamic acid, histidine, arginine, lysine, and glycine copper salts are insoluble in anhydrous methanol, but are soluble in water.

C. The copper salts of leucine, phenylalanine, and aspartic acid are insoluble in cold water and in dry methanol.

7. PRECIPITATION OF AMINO ACIDS AS THE BARIUM CARBAMATES (KINGSTON AND SCHRYVER, 365, 570, 571)

Principle: The alternate addition of $Ba(OH)_2$ and CO_2 to an ice cold solution of amino acids results in the formation of the barium carbamates. These can be precipitated by the addition of 2 to 3 volumes of ethanol. All amino acid carbamates, except that of glycine, are soluble in cold water.

8. FRACTIONATION OF CARBAMIDO ACIDS (BOYD, 118)

Principle: A neutralized solution of amino acids is refluxed with an excess of KOCN to form the carbamido compounds. The reaction of the solution is brought to pH 4 with H_2SO_4 and the insoluble carbamido derivatives of leucine, isoleucine, and phenylalanine are removed. The carbamido derivatives of the monoamino monocarboxylic acids are soluble in 80 per cent alcohol.

9. SEPARATION OF AMINO ACIDS BY MEANS OF DRY FATTY ACIDS (PRZYLECKI AND KASPRZYK, 531)

Principle: The protein hydrolysate is freed from all inorganic ions and the solution is evaporated to dryness. It is claimed that the basic amino acids are soluble in anhydrous butyric and caproic acids; that glycine, alanine, valine, leucine, phenylalanine, proline, and hydroxyproline are soluble in 99 to 99.5 per cent acetic and propionic acids, while tyrosine, cystine, aspartic, and glutamic acids are insoluble.

Comment: The authors' experience with this procedure has not been encouraging.

*10. DETERMINATION OF AMINO ACIDS BY THE SOLUBILITY PRODUCTS OF THEIR SALTS (BERGMANN *et al.* 70, 315, 197, 588, 465A)

Principle: The solubility product of an amino acid salt is a constant. The essential requirement of the solubility method is that the protein hydrolysate and the precipitating reagent should be agitated and filtered under constant temperature conditions.

Apparatus: cf. Diagram II.

Method: (Ing and Bergmann, 315): The reaction mixture is placed in the small bottle (a) above which rests an inverted sintered glass funnel (b). Both the bottle and the funnel have been weighed. The whole apparatus is enclosed in a centrifuge tube (c) which is closed by a tightly fitting rubber stopper (d).

The apparatus is shaken in ice water for 24 to 48 hours after which the precipitate is removed by inverting the apparatus and

* Recommended procedure.

centrifuging at 2500 to 3000 R.P.M. for 5 to 10 minutes. The tube must be kept at 0° during the entire operation.

After centrifuging, the bottle and funnel are removed, carefully wiped, and weighed at once. They åre then dried to constant weight in a desiccator. The loss of weight on drying enables a correction to be made for the solids contained in the mother liquor adhering to the precipitate and the apparatus. The solids in the original solution must be known. The correction for adhering solids may be checked by washing the precipitate at 0° with a cold saturated solution of the pure amino acid salt.

This process is carried out with an aliquot of the hydrolysate to which an excess of the amino acid salt has been added and with a second solution containing the same reagents as the first, but with R moles of precipitating reagent added to the hydrolysate before the introduction of the amino acid salt.

The undissolved amino acid salt is removed and weighed.

DIAGRAM II
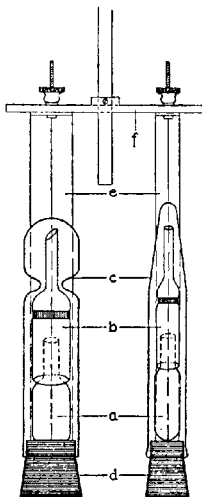
FIG. 1. Two different sizes of apparatus for the analysis of the amino acid content of protein. *a* represents a small bottle; *b*, an inverted sintered glass funnel; *c*, a centrifuge tube; *d*, a tightly fitting glass stopper; *e*, an angle brass which depends from a metal disk, *f*. (From: H. R. Ing and Max Bergmann, The Journal of Biological Chemistry, Vol. 129, No. 2, August, 1939.)

Calculations: The quantity of the amino acid present in the hydrolysate is calculated from the following equations.

I. if $K_1 = K_2$, then

$$A = \frac{S^2 - S_2(R + S_2)}{(R + S_2) - S_1}$$

II. if $K_1 \neq K_2$ but if $K_1 = FK_2$, then

$$A = \frac{S_1^2 - FS_2(R + S_2)}{F(R + S_2) - S_1}$$

where $K_1 = S_1(A + S_1)$

$K_2 = (R + S_2)(A + S_2)$

and $A = mM$ of Amino Acid in sample

$R = mM$ of Reagent added

$S = mM$ of Salt dissolved.

Comment: It is necessary to ascertain that the precipitating reagent does not precipitate any other amino acid under the conditions used in the experiment. The solubilities of the unnatural and racemic amino acid salts are not the same as the natural salts. This adds a further complication to the method.

*11. THE ISOTOPE DILUTION METHOD (USSING, 626, RITTENBERG AND FOSTER, 547)

Principle: A compound which has an abnormal isotope content is inseparable from its normal analogue by the usual laboratory procedures.

The quantity of an amino acid in a protein hydrolysate can be determined by the following formula.

$$Y = \left(\frac{C_0}{C} - 1\right)X$$

where $Y =$ amino acid in the protein hydrolysate

$X =$ added amino acid containing C_0 per cent of isotope

and $C =$ the isotope content of the isolated amino acid.

C_0/C should be between 5 and 10.

Because of the fact that optical isomers have general solubilities different from those of racemic compounds or mixtures; the protein hydrolysate must be entirely racemized, or the racemic synthetic amino acid containing the isotope must be resolved and the natural amino acid alone used, or the racemic isotope can be added to the hydrolysate and a sample of the natural isomer *only* is isolated.

The errors of this method are due to the purity of the compound

* Recommended procedure.

added, the purity of the amino acid isolated, and the accuracy of the isotope analysis. The errors of isotope analyses in competent hands are very low, while the purity of the samples of amino acid added and isolated can be easily checked by repeated crystallization and isotope determination.

Comment: This procedure, which appears to be the most accurate method for the determination of amino acids in protein hydrolysates, is limited only by the availability of the complicated and expensive apparatus needed, the synthetic isotope containing amino acids, and expert technical ability.

12. SEPARATION OF AMINO ACIDS BY CHROMATOGRAPHIC ADSORPTION

A. Separation on Activated Carbon (Tiselius, 609, 610, 611, cf. 667)

Principle: If an aqueous 0.5 per cent solution of amino acids in 0.1 M NaCl is allowed to pass upward very slowly through a layer of activated carbon, the different solutes will become more or less retarded as compared to the solvent. The degree of retardation will depend upon the degree of adsorption. The concentration of an amino acid in the effluent can be measured by the refractive index and mixtures of amino acids may be separated from each other by this technique.

B. Separation on Activated Titania (Strain, 594)

Principle: Activated TiO_2, prepared from $TiCl_4$ and K_2CO_3, will adsorb glutamic acid at pH 3.2 and histidine at pH 10.

C. Separation of Polyamino and Polycarboxylic Amino Acids by Ion Exchange Substances (Block, 107, Cannan, 148)

Principles: The diamino acids are removed from protein hydrolysates by treatment with cation exchange synthetic zeolites such as the sulfonated resins, Amberlites IR-1 and IR-100 or the sulfonated coal, Zeo-Karb (cf. Chapter I).

The dicarboxylic amino acids can be separated from cation-free protein hydrolysates by the anion exchange substances, Amberlite IR-4 or De-Acidite (cf. Chapter VI).

Method: 1. Polyamino Acids. If an ammonia-free protein hydrolysate is allowed to flow at a rapid rate through a cation exchange substance such as the resins, Amberlite IR-1 or IR-100 (Resinous Products Co., Philadelphia) or a sulfonated coal such as Zeo-Karb (Permutit Company, New York City), arginine, histidine, and lysine are quantitatively removed from the solution and the pH of the effluent rises. The residual mono- and dicarboxylic

amino acids are removed from the column by thorough washing with cation-free water.

Lysine is separated by elution with 1.5 to 2.5 per cent H_2SO_4. A portion of the adsorbed arginine and histidine is also eluted at this step.

Histidine is removed from the column by ion exchange with 1 per cent NH_4OH. The ammonia is eluted by washing the column with 1 per cent HCl and finally the arginine is removed by elution with either 7 per cent HCl or 5 per cent H_2SO_4.

2. Dicarboxylic Amino Acids. The cation-free protein hydrolysate is passed through a column of an anion exchange synthetic zeolite such as De-Acidite or Amberlite IR-4. The column is thoroughly washed with anion-free water, and glutamic and aspartic acids are quantitatively removed by exchange with 5 per cent HCl, 4 per cent H_2SO_4 or 2 per cent Na_2CO_3. The solution of HCl or H_2SO_4 is passed over the column of IR-4 until the pH of the effluent is below pH 1, the solution of Na_2CO_3 is passed over the column until the pH of the effluent rises to 8.2 or higher.

This exchange reaction, in contrast to the method of acid elution used for the removal of the polyamino acids, is, as expected from theoretical considerations, quantitative.

3. Monoamino Monocarboxylic Acids. The effluent from the anion and cation exchange adsorptions consists of monoamino monocarboxylic acids. This colorless solution is most suitable for the determination of the monoamino acids by the methods described in this monograph and is especially valuable for the preparation of these substances by any of the standard methods.

D. Separation of Amino Acids by Partition Chromatography (Gordon, Martin, and Synge 261, 261A, 261B, 261C, 261D, 439 and Catch, Cook, and Heilbron, 150).

Principle: A solution of acetylated amino acids is chromatographed from an organic solvent ($CHCl_3$-C_4H_9OH, etc.) onto a column consisting of a water-retentive support such as activated silica gel or Hyflo-Supercel. The column is saturated with bound water (Martin) or with an inorganic base such as an hydroxide or carbonate for acidic amino acids or a weak inorganic acid such as $NaHSO_4$ for the extraction of the diamino acids (Catch).

Reagents: Preparation of Silica Gel (261A). Commercial water glass (140 Tw.-Jos. Crosfield, Ltd., Warrington, England) is diluted with 3 volumes of distilled water containing a little methyl orange. 10 N HCl is added in a thin stream with vigorous stirring, addition being interrupted at intervals and stirring continued to

get efficient mixing. The solution changes first slowly, then rapidly to a thick porridge and all but the smallest lumps are broken up by stirring. When the mixture is permanently acid to thymol blue, addition of HCl is stopped and the mixture is left 3 hours. It is filtered on a Buchner funnel and washed with distilled water (approx. 2 L/250 gm. dry gel) without allowing the cake to crack. The gel is then suspended in $N/5$ HCl and aged 2 days at room temperature. It is again filtered and washed in the same way with distilled water (approx. 5 L/250 gm. dry gel) until the washings are free from methyl orange. Finally, the gel is crumbled and dried at 110°C in an air oven. With such a preparation the addition of 53 per cent W/W of water saturated with methyl orange or a 0.05 per cent aqueous solution of pelargonin chloride, should be satisfactory.

Preparation of Column (439, 261A). 3 gm. of silica gel saturated with methyl orange are suspended in 3 per cent n-butanol-chloroform (3 ml. of BuOH-100 ml. of CHCl₃). This suspension is introduced into a chromatogram tube having an internal diameter of 1 cm., 30 cm. long, with a double layer of filter paper or a perforated silver disc at the bottom. The solvent is allowed to drain out, but a stopper is placed in the top of the tube to prevent the entrance of air. The solvent emerging is almost devoid of indicator for the MO should be firmly held in the aqueous phase.

Hydrolysis and Acetylation (261D). The protein is hydrolyzed with 6 N HCl in sealed tubes *in vacuo* for 24 hours. After removing the excess HCl by concentration, the residue is dissolved in a little water and 6 N NaOH is added to alkaline to thymolphthalein, the solution is concentrated to a thin syrup. The amino acids are acetylated by 5 ml. of 4 N NaOH plus 1 ml. of acetic anhydride added in 5 equal portions in the course of 15 minutes. The solution is shaken and cooled in ice water between each addition. The solution is allowed to remain alkaline to thymolphthalein for 10 minutes and then it is acidified to thymol blue with 10 N H₂SO₄.

Chromatographic Procedure (439, 261A). When the column is ready the acetylated amino acid mixture is transferred to it by repeated hot extraction with 1 ml. portions or less of the solvent mixture. The solution is added carefully to the top of the column from a pipette without disturbing the gel. Each portion is allowed to drain into the gel before the next is added. An alternative procedure is to adsorb the acidified acetylation mixture on sufficient silica gel, then make a slurry with 17 per cent butanol-chloroform (half-saturated with water) and make into a column in the usual way (*cf.* 261D).

The acetylated amino acids are chromatographically separated by repeated adsorption on silica gel and elution with the following solvents: 1 per cent butanol-CHCl₃, 3 per cent butanol-CHCl₃, 17 per cent butanol-CHCl₃, 5 per cent propanol cyclohexane, 30 per cent propanol-cyclohexane.

Comments: This procedure, which permits the separation and estimation of a half dozen mono amino acids from a solution containing only 2 to 4 mg. of nitrogen, appears to represent a marked advance in amino acid chemistry, although the technical details are still in a state of flux. The table given below lists some of the results obtained by Gordon, Martin and Synge using partition chromatography. The readers attention is called to the isolation of ornithine from tyrocidine, a unique finding of considerable importance.

Amino Acid Composition Calculated to 16.0 gm. of N.
(261A, 261B, 261C, 261D)

AMINO ACID	GELATIN	WOOL	GRAMICIDIN	TYROCIDINE
	gm.	gm.	gm.	gm.
Methionine	0.5–1.1	0.3		
Tryosine		5.8		12.4–14.1
Tryptophane			47–53	1–11
Phenylalanine	0.8–1.9	1.5		23–28
Leucine				
Isoleucine	5.9	9.3	30.3	11–13
Valine	2.3	5.2	22.2	9–11
Glutamic Acid				10.3–12.1
Aspartic Acid				9.3–10.5
Proline	12.7	6.8		7.7– 8.5
Alanine	8.4–9.2	4.0	10.3	
Glycine			4.5–5.7	
Ornithine				7–10

E. Microbiological Determination of Amino Acids (Lyman, Kuiken, Norman, and Hale, 433B; Thompson 608A)

Principle: The ability of certain microorganisms to grow on synthetic media permits the development of methods for the quantitative determination of each separate constituent in the medium. This procedure has been widely used during the past few years for the determination of vitamins and recent reports indicate that it may be more accurate and much simpler than many of the chemical methods for amino acids available at present. The development of this method is awaited with interest.

Procedure: (Lyman, Kuiken, Norman, and Hale, 433B). The complete medium for *Lactobacillus arabinosus* 17–5 of Snell and Wright (582A) is used except the casein hydrolysate is replaced by

2 mg. each of threonine, valine, leucine, isoleucine, lysine, phenyl-alanine, alanine, arginine, histidine, proline, serine, methionine, tyrosine and 4 mg. of aspartic acid and 4 mg. of glutamic acid. 1 mg. of a Norite eluate from tomato juice per 10 ml. of medium and p-aminobenzoic acid are also added.

By leaving out one of the amino acids which is essential for the growth of *Lactobacillus arabinosus* (glutamic acid, tryptophane, threonine, valine, leucine, isoleucine, cystine, lysine or phenyl-alanine), a medium for the determination of that particular amino acid is prepared. The amount of lactic acid formed in the test cultures is indicative of the quantity of the amino acid present in the unknown.

Comment: The Food Research Laboratories, Long Island City, New York is prepared to carry out amino acid analyses by the microbiological method.

CHAPTER IX

PART III

CARBOHYDRATE REACTIONS

1. Dische's Test for Carbohydrates (196)

Principle: Dische (196) found that the carbohydrate component of thymonucleic acid gives colored compounds with (a) 0.5 per cent HCl and indole; (b) carbazole in 80 volume per cent of H_2SO_4, (c) diphenylamine in H_2SO_4.

A. Gurin and Hood's Elaboration of the Dische Reaction (274)

Principle: Dische's carbazole reaction gives a pink color with glucose, a brown color with mannose, and an intermediate color with galactose.

Reagents: Purification of Carbazole. Dissolve 1 part of technical carbazole in 20 parts of diluted H_2SO_4 (8 volumes of nitric acid-free H_2SO_4 to 1 volume of H_2O). Stir for 1 hour. Pour the solution into a large volume of ice water, filter and wash the carbazole with cold water. Recrystallize the precipitate first from toluene, then from 70 per cent ethanol. Repeat the whole process starting with solution in H_2SO_4.

Method: Cool 9 ml. of 8:1 H_2SO_4 to 0° and add 1.0 ml. of unknown containing 0.05 to 0.2 mg. of sugar. Do not allow the temperature of the solution to rise above 0° during this addition. Then add 0.3 ml. of 0.5 per cent carbazole in absolute ethanol. Mix and heat in a boiling water bath for 10 minutes. Cool to 0° and read the color.

If the sugar solution is pure, use filter 660 mu for glucose, 540 mu for galactose, 520 mu for mannose, and 420 mu for fructose.

Comment: The quantities of two sugars in a mixture can be estimated from the ratio of the extinction coefficients at 420 mu and 520 mu respectively, provided other supplementary qualitative tests are carried out.

Tryptophane introduces a serious error in this reaction.

Gurin and Hood (274) found 0.5 per cent of galactose in casein.

2. The Pauly-Ludwig Hexosamine Reaction (515)

Principle: Hexosamines condense with acetylacetone to yield substituted pyrrols. The latter give colored compounds when treated with p-dimethylaminobenzaldehyde (Elson and Morgan 208; Sørensen, 583).

297

$$\text{Glucosamine} + CH_3COCH_2COCH_3 \rightarrow CH_2OHCHOHCHOHCHOHC \quad \underset{NH}{\overset{\displaystyle CH\text{---}C\cdot COCH_3}{\underset{\diagdown\diagup}{\overset{\|\qquad\|}{}}}} C\cdot CH_3$$

3-acetyl-2-methyl-5-tetrahydroxybutylpyrrol.
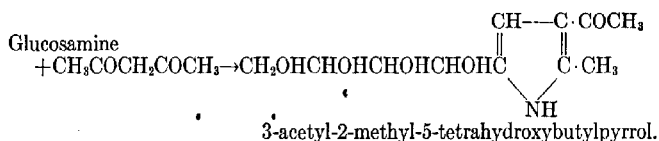
A. Palmer, Smyth, and Meyer's Modification of the Method of Elson and Morgan (208, 510)

Reagents: Acetylacetone. Prepare just before use by dissolving 0.2 ml. of acetylacetone in 10 ml. of 0.5 N Na_2CO_3. Keep cold.

Aldehyde-free Alcohol. Distill ethanol after treatment with Ag_2O and NaOH in an all glass still.

Purification of p-Dimethylaminobenzaldehyde: Dissolve the best commercial grade in concentrated HCl, dilute with water and fractionally precipitate the aldehyde by the addition of saturated sodium acetate. Use only the pure white fractions.

Ehrlich's reagent: Dissolve 800 mg. of purified p-dimethylaminobenzaldehyde in 30 ml. of aldehyde-free ethanol and add 30 ml. of concentrated HCl. Keep cold.

Method: 1. Hydrolysis. A sample containing 0.25 to 1.25 mg. of hexosamine is diluted to 1 ml. with water and 1 ml. of 8 N HCl is added. The tube is sealed off and placed in a boiling water bath for 8 hours. The contents are then diluted to 25 ml. Ten ml. of this solution are titrated to the turning point of methyl red with 0.25 N NaOH.

Another 10 ml. of the hydrolysate are transferred to a 25 ml. volumetric flask and neutralized with 98 to 99 per cent of the amount of alkali determined from the preliminary titration. Efficient stirring is necessary at this point. The solution is diluted to 25 ml.

2. Condensation with Acetylacetone. 0.5 ml. of acetylacetone are added to 1 ml. of the neutralized hydrolysate. The tube is placed in a boiling water bath for 15 minutes, so that the contents are covered by the boiling water, but the neck of the tube can be cooled by a strong current of air.

The tubes are then cooled in water.

3. Reaction with p-Dimethylaminobenzaldehyde. The solution is diluted with 2.5 ml. of aldehyde-free alcohol and 0.5 ml. of Ehrlich's reagent are added with stirring. The solution is then diluted to 5 ml. with alcohol and the contents are mixed. The color is read using filter 530 mu.

Comment: It is important to carry out both reagent "blanks" and controls with standard solutions simultaneously with each group of unknowns. The standard conditions must be adhered to in every detail.

CHAPTER X

SUMMARY TABLES

THE values given in the following tables were taken from the
detailed figures in the previous Chapters and are presented
by the authors with considerable reserve for these figures can
be easily misinterpreted.

It cannot be stressed too often that, with few exceptions, the
methods for the estimation of the amino acids in protein hydrol-
ysates are not ideal; there are further errors of varying magnitude
during the liberation of the amino acids by hydrolysis, and finally
in the case of the nutritionally important food proteins, especially
those of plant origin, large specie differences may exist. In fact
just as the vitamin and mineral contents of a plant can be varied
by breeding and cultivation, it is probable that the amino acid
composition of the proteins of that plant can likewise vary. There
may come a day, in the not too distant future, when plants will be
bred for their content of the essential amino acids as well as certain
of the vitamins.

As all the data in previous Chapters have been calculated as
gm. of amino acid per 16 gm. of nitrogen, the values given in the
summary tables are likewise so calculated. If, for example, one
wishes to know the approximate amino acid composition of a puri-
fied laboratory sample of fibrin which analyzes for 17.7 per cent
of nitrogen on a moisture and ash-free basis, then it is only neces-
sary to multiply the proper values in Table 2 by the factor

$$\frac{17.7}{16.0} \quad \text{or} \quad 1.11.$$

If on the other hand, one wishes to know approximately how
much of each of the essential amino acids is being ingested by a
group of rats eating a diet which contains commercial fibrin,
N = 13.0 per cent; then these values in Table 2 are multiplied by
the factor

$$\frac{13.0}{16.0} \quad \text{or} \quad 0.81.$$

If a sample of flour contains 12 per cent protein (N×5.7), then
the approximate quantity of the amino acids supplied by 100 lbs.

of that flour could be ascertained by multiplying the values in the last column of Table 10 by the factor

$$\frac{2.1}{16.0} \text{ or } 0.131.$$

TABLE 1

Approximate Percentage of Amino Acids in Animal Proteins
Albuminoids
Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	GELATIN	ELASTIN
Arginine	9.3	0.9
Histidine	1.0±0.1	0.0
Lysine	5.0	?
Tyrosine	0.2	1.5
Tryptophane	0.0	0.0
Phenylalanine	2.5	3.4
Cystine	0.1	0.2
Methionine	0.8	0.4
Serine	3.3	
Threonine	1.5	2.5
Leucine	3.7±0.5	28[a]
Isoleucine	1.7	
Valine	2.5	13
Glutamic Acid	10.2	
Aspartic Acid	6.2	0.0
Glycine	23.6	27.5
Alanine	10	6
Proline	15.3±0.4	14.2
Hydroxyproline	13	1.9

[a] Includes isoleucine.

TABLE 2

Approximate Percentage of Amino Acids in Animal Proteins
Blood Proteins
Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	FIBRIN	HEMOGLOBINS	SERUM PROTEINS	BENCE-JONES PROTEINS
Arginine	7.8	3.5±0.3	5.8±0.3	4-6
Histidine	2.9	7.6±0.4	2.6±0.1	1
Lysine	8.8	9.0	8.0±0.4	4-7
Tyrosine	5.1±0.7	2.4±0.3	5.4±0.3	5-10
Tryptophane	3.4±0.4	1.5	1.7±0.1	1-2
Phenylalanine	7	7.7±0.9	5.4	
Cystine	1.9±0.4	0.4-1.8	3.6±0.1	3
Methionine	3.1	0.5-1.8	1.9	0.7
Serine		5.2		
Threonine	7.9	6.8	6.3	
Leucine	14.3±3.9	16.6±2.3	18	
Isoleucine	5.0±0.5	1.5±0.3	3	
Valine	3.9±1.8	8.2±1.0	6	
Glutamic Acid	13.8±0.1	5.7		8.6
Aspartic Acid	11.9±0.1	8.1		4.7
Glycine	5.4±0.1	trace		
Alanine		7-8		5
Proline		2		7
Hydroxyproline		0	0	0

TABLE 3
Approximate Percentage of Amino Acids in Animal Proteins .
Egg Proteins
Calculated to 10.0 gm. of Nitrogen

AMINO ACIDS	ALBUMIN	VITELLIN	LIVETIN	EGG WHITE	WHOLE EGG*	EGG YOLK
Arginine	5.7±0.3	8.0	5.8	5.8	6.4	8.2
Histidine	2.4	1.4	1.2	2.2	2.1	2.6
Lysine	7.7	6	5.2	6.5	7.2	5.5
Tyrosine	4.2±0.1	5.3	6.3	4.8	4.5	5.3
Tryptophane	1.4±0.2	1.8	1.5	1.6±0.2	1.5	1.6±0.2
Phenylalanine	6	3		5.5	6.3	5.7
Cystine	2–3	1.3	3.1	2.3±0.4	2.4	1.9
Methionine	5.0±0.3	2.9	2.4	4.4	4.1	2–3
Serine	7.6	9.4				
Threonine	3–4	4.9			4.9	
Leucine	9.4				9.2	
Isoleucine					8.0	
Valine	6.8				7.3	
Glutamic Acid	16.3±0.2	12.7	7.0			
Aspartic Acid	8.2±0.2		3.1			
Glycine	1.9	1			2.5	
Alanine	7.4		6			
Proline	4–5		2–3			

* Considered by the authors to represent the best balanced protein.

TABLE 4
Approximate Percentage of Amino Acids in Animal Proteins
Feeds and Foods
Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	TANKAGE	MEAT SCRAPS	FISH STICK WATER	FISH MEAL
Arginine	5.5	7.0	5.4	7.4
Histidine	2.7	2.0	2.6	2.4
Lysine	6.0	5.1	4.1	7.8
Tyrosine	2.9	3.2	0.8	3.6
Tryptophane	0.7	0.7	0.8	1.2
Phenylalanine	6.0	4.5	1.9	4.8
Cystine	1	1.0	+	1.0
Methionine	3	2.0	1.5	2.9
Threonine	3–4	4	2.3	5.1
Leucine	13	8.0	2	7.1
Isoleucine	2–3	6.3	1	6.0
Valine	6	5.8	3	5.8

TABLE 5

Approximate Percentage of Amino Acids in Animal Proteins
Hormones and Enzymes
Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	INSULIN	PEPSIN	THYROGLOBULIN
Arginine	3.3	1.4	7.4
Histidine	5.0	trace	3
Lysine	1.3	1.9	5–6
Tyrosine	12.8	10.8	3.0
Tryptophane	0.0	2.3	2.1
Phenylalanine	8.4		
Cystine	12.9±0.4	1.5	2–4
Methionine	0.0		1.3
Serine	3.6		
Threonine	2.7	9.9	

TABLE 6

Approximate Percentage of Amino Acids in Animal Proteins
Keratins
Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	HAIR	WOOL	HORN	FEATHERS	SILK FIBROIN
Arginine	10.7	10.0	10.4	6.5	0.9
Histidine	1.0	0.7	1	0.7	0.06
Lysine	2.6	3.0	3.2	1.8	0.5
Tyrosine	3.1	5.1	4–6	2.3	11.1
Tryptophane	1.3	1.5	1.5	1	
Phenylalanine	2.7	3.9	4	5	
Cystine	15.9±0.9	11.1±0.9	7.3	9–10	0
Methionine	1–2	0.6			0
Serine					12.9
Threonine	6.4		5–6		1.3
Leucine	7–10	11[a]	15±1.6		0.8
Isoleucine	3–4		4–5		
Valine	3–6	4–5	5±0.5		2.7
Glutamic Acid	12.2	15.3	18		
Aspartic Acid	3.0	7.3	3		
Glycine	4–5	7	10		36.8
Alanine		4	2		22.2
Proline	8.5	9.3			1

[a] Includes isoleucine.

TABLE 7

Approximate Percentage of Amino Acids in Animal Proteins

Milk Proteins

Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	CASEIN	LACTALBUMIN	β-LACTO-GLOBULIN	WHOLE MILK COW	WHOLE MILK* HUMAN
Arginine	4.1±0.2	3.5±0.5	3.1	4.3	5.0
Histidine	2.5±0.3	2.0±0.3	1.8	2.5	2.7
Lysine	6.9±0.7	9.0	10.4	7.5	7.2
Tyrosine	6.4±0.4	5.3±0.1	4.3	5.3	5.1
Tryptophane	1.8±0.2	2.3±0.3	2.0	1.6	1.9
Phenylalanine	5.2±0.5	5.6	5.3	5.7	5.9
Cystine	0.36±0.04	4.1	3.6	0.7	3.4
Methionine	3.5±0.3	2.8±0.2	3.6	3.7	2.0
Serine	7.5	4.9	4.6		
Threonine	3.9±0.1	5.3	6.0	4.6	4.6
Leucine	12.1	15ᵃ	17.7±4.2	11.3	10.2
Isoleucine	6.5		6.6	6.2	7.6
Valine	7.0	4	6.2	6.6	9.9
Glutamic Acid	22.8		22.1		
Aspartic Acid	6.3		10.1		
Glycine	0.5				
Alanine	5.6	0–1			
Proline	8.2				
Hydroxyproline	2				

ᵃ Leucine and isoleucine.

TABLE 8

Approximate Percentage of Amino Acids in Animal Proteins

Tissue Proteins

Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	ENTIRE ANIMALS	BRAIN	LIVER	ANIMAL MUSCLE	FISH MUSCLE	MIXED GLANDS
Arginine	6.6±0.4	6.6±0.2	6.5±0.7	7.2±0.9	7.4	6.6±0.4
Histidine	2.0	2.6±0.2	2.6±0.3	2.1±0.2	1.9±0.6	2.2±0.4
Lysine	6.0±0.3	6.2±0.3	6.3	7.6±1.0	7.8	5.7±0.5
Tyrosine	3.3±0.3	4.1±0.1	3.9±0.2	3.1±0.3	3.6	3.1±0.5
Tryptophane	1.0±0.2	1.3±0.1	1.5±0.2	1.2±0.2	1.3±0.1	1.0±0.3
Phenylalanine	4.0	4.9±0.5	7.3	4–5	4.8	4.6±0.9
Cystine	2.2±1.0	1.8±0.2	1.4±0.1	1.1±0.3	1.2±0.1	1.3±0.1
Methionine	3	3	3.2±0.1	3.2±0.3	3.2	3
Serine		7.1	7.3	5.7	4.5	6.8
Threonine	4.5	5.8	5.8	5.3±0.4	5.1	3.8±0.3
Leucine	10.8±0.1	13.4±2.2	8.4	8.0	7.1	8.0
Isoleucine		3.6±0.3	5.6	6.3	6.0	5.6
Valine		4.9±0.7	6.2	5.8	5.8	5.3
Glutamic Acid			11.4	15	14	
Aspartic Acid			6.9			
Glycine	10		8.5	4		
Alanine		6			7	
Proline				6	3	

TABLE 9

Approximate Percentage of Amino Acids in Plant Proteins

Corn Proteins

Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	WHOLE CORN	GERM	GLUTEN	ZEIN	ZEIN RESIDUE	ALBUMINS
Arginine	4.0	6.8	3.1	1.6±0.2	2.9	5.4
Histidine	2.4	2.7	1.7	0.9±0.2	1.6	6.7
Lysine	2.0	5.8	1.1	0.0	1.6	1
Tyrosine	6.1	4.9	6.2	5.0±1.2	6.2	3.8
Tryptophane	0.8	1.3	0.6	0.1	1.1	0.7
Phenylalanine	5.0	5.6	6.6	6.4±0.7	4.5	1.7
Cystine	1.1	1.2±0.3	1.2	0.8±0.1	1.8±0.3	0.5
Methionine		2.3	4	2.0	4.8	
Threonine	3.6	4.4	4.0	2.4	4.0	3.9
Leucine	21.5±2.4	6.7	24.7±3.7	23.7±2.1	11.0±2.9	11.3±4.1
Isoleucine	3.6±0.3	3.7±0.4	4.9±0.3	4.3±0.4	2.0±0.3	1.3±0.4
Valine	4.6±0.7	5.8±1.2	4.6±1.4	2.4±0.9	5.5±1.0	2.5±1.1
Glutamic Acid			24.5±0.4	35.6		
Aspartic Acid				3.4		
Glycine			4.3	0.0	9.6	
Alanine				9.9		
Proline				9–12		
Hydroxyproline				1		

TABLE 10

Approximate Percentage of Amino Acids in Plant Proteins

Grasses, Leaves, Yeasts, Enzymes, Viruses

Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	GRASSES	LEAVES	VIRUSES	YEASTS
Arginine	6.9±0.4	6.6±1.0	9–10	4.3±0.5
Histidine	1.5±0.4	1.4±0.3	0–0.6	2.8±0.3
Lysine	5.8	5.0±0.4	0.0	7.5±0.4
Tyrosine	5.0±0.2	5.3±0.2	4–6	4.2±0.2
Tryptophane	2.1±0.1	2.1±0.4	1.5 to 4.9	1.3±0.2
Phenylalanine		4–5	4–10	4.1±0.5
Cystine	2.0±0.1	1.8±0.2	0.7	1.1
Methionine	2.4±0.1	2.3±0.3	0.0	2.0
Serine			7.0	
Threonine		4.8	6.3	5.5
Leucine		11		7.3
Isoleucine		5.4±0.2		6.0
Valine		6		5.3
Glutamic Acid			6.0	
Aspartic Acid			3.0	
Glycine			0.0	
Alanine			2.8	
Proline			5.5	
Hydroxyproline				

TABLE 11

Approximate Percentage of Amino Acids in Plant Proteins

Proteins of Seeds

Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	COTTON-SEED MEAL	LINSEED MEAL	PEANUT FLOUR	SOYBEAN MEAL	OATS	RICE
Arginine	7.4	6.9	9.9	5.8	6.0	7.2
Histidine	2.8	1.9	2.1	2.3	2.0	1.5
Lysine	2.7	2.0	3.0	5.8	3.3	3.2
Tyrosine	3.2	5.1	4.4	4.1	4.5	5.8
Tryptophane	1.3	1.6	1.0	1.6	1.3	1.3
Phenylalanine	6.8	5.8	5.4	5.7	6.9	6.3
Cystine	2.0	1.9	1.6	0.6±1.4	1.8	1.4
Methionine	1.6	2.3	1.3	2.0	2.3	3.4
Serine						
Threonine	3.0	4.5	1.5	4.0	3.5	3.9
Leucine	5.0	7.5±2.8	5.5	6.6	8.0	9.0
Isoleucine	3.4	3.4±0.3	3.4	4.7	5.3	5.1
Valine	3.7	5.8±1.3	4.0	4.2	6.5	6.4
Glycine	5.3		5.6			

TABLE 12

Approximate Percentage of Amino Acids in Plant Proteins

Wheat Proteins

Calculated to 16.0 gm. of Nitrogen

AMINO ACIDS	WHOLE WHEAT	GERM	GLIADIN	GLUTEN	FLOUR
Arginine	2.8±0.5	6.0	2.6±0.2	3.9	3.9
Histidine	1–2	2.5	1.6±0.2	2.2	2.2
Lysine	2.7	6.4	0.7	1.9	1.9
Tyrosine	3.8±1.0	3.8	2.8±0.4	3.8	3.8
Tryptophane	1.2	1.0	0.8±0.1	1.0	0.8
Phenylalanine	5.7	4.2		5.5	5.5
Cystine	1.3±0.3	0.6	2.1±0.2	1.7	1.9
Methionine	2.0	2	2.1	3	3
Serine					
Threonine	3.3	3.8	2.7	2.5	2.7
Leucine	5.8	7.4±2.3	6.1		9.1
Isoleucine	3.3	3.0±0.5			4.5
Valine	3.6	4.1±1.0			5.0
Glutamic Acid			42.	27	
Aspartic Acid			1.3	10	
Glycine			0–1	9	7.2
Alanine			2–3	5	
Proline			12	10	
Hydroxyproline					

THE ESSENTIAL AMINO ACID REQUIREMENTS OF MAN

THE data summarized in this monograph have been used to estimate the average annual human consumption of each of the essential amino acids by the people of the United States during the years 1937 to 1941 inclusive (*cf.* Block, 108). Table I gives the average annual per capita consumption of foodstuff, of protein, and of each of the essential amino acids.

Table II indicates the optimal daily essential amino acid requirements of man as calculated from the data of W. C. Rose, on growing rats, and the analyses of I. G. Macy and Block (*cf.* 108). The quantities of each of these amino acids which would be supplied by the ingestion of 100 gm. of protein from meat, milk, white flour, and bread enriched with 6 per cent milk solids are also presented.

Table III shows the average requirement of each of the essential amino acids as based on the figures of Rose, Macy, and Block and the *percentage* of each of these amino acids which would be supplied by 100 gm. of protein from meat, milk, flour, and bread. Even though cow's milk protein is definitely inferior to the protein of human milk, the calculations in table III demonstrate its average superiority over meat, flour, and bread proteins.

The deficiencies of white flour in lysine and tryptophane are well known, but not those of threonine and valine. A brief calculation may be of interest. If the daily human requirement of lysine is 5.2 gm. then it would take 186 gm. of protein or approximately 6200 calories from enriched bread made with 6 per cent milk solids to supply 5.2 gm. of lysine per day. If the bread were made from a "lean" formula, 260 gm. of protein or approximately 8600 calories of bread (8 loaves) would have to be eaten per day.

However, it appears from these calculations that the addition of 2 gm. of lysine per 100 gm. of protein in bread prepared with 6 per cent milk solids or 2.4 gm. of lysine per 100 gm. of protein of bread made with 3 per cent of milk solids, would supply the average daily requirement of the essential amino acids if 108 gm. of protein or approximately 3600 calories of bread (3 loaves) were eaten.

TABLE I

Estimated Average Annual Per Capita Consumption of Essential
Amino Acids in the United States 1937–1941

	DAIRY PRODUCTS	MEATS AND FISH	EGGS	BEANS AND NUTS	CEREALS	TOTAL POUNDS	GRAMS PER DAY
	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	
As Eaten*	307.8	136.5	37.7	16.0	196.1		
As Protein	14.9	27.3	5.7	4.0	19.6	71.5	89
Arginine	0.64	1.80	0.40	0.21	0.76	3.81	4.74
Histidine	0.37	0.57	0.14	0.07	0.45	1.60	1.99
Lysine	1.12	2.10	0.34	0.19	0.40	4.15	5.16
Tyrosine	0.79	0.96	0.28	0.16	0.91	3.10	3.86
Tryptophane	0.24	0.33	0.09	0.06	0.18	0.90	1.12
Phenylalanine	0.85	1.23	0.32	0.23	1.16	3.79	4.71
Cystine	0.16	0.30	0.12	0.04	0.33	0.95	1.18
Methionine	0.42	0.93	0.23	0.08	0.69	2.35	2.93
Threonine	0.69	1.17	0.29	0.16	0.61	2.92	3.63
Leucine	2.24	3.3	1.1	0.32	3.14	10.1	12.6
Isoleucine	0.75	0.93	0.30	0.16	0.81	2.95	3.67
Valine	0.97	0.95	0.28	0.18	0.75	3.13	3.90
Glycine	0.07	1.4	0.15	0.06		1–2	

Cereals: calculated from ⅔ wheat and ⅓ corn.
* From Agricultural Situation, August 1942.

TABLE II

Daily Essential Amino Acid Requirements of Man
Calculated Amounts. Sources of Supply

AMINO ACID	Suggested by			SUPPLIED BY 100 GM. OF PROTEIN FROM			
	Rat Growth (Rose)	Amino Acid Analyses		Meat	Milk	White Flour	"Enriched"* Bread
		(Macy)	(Block)				
	gm./day	gm./day	gm./day	gm.	gm.	gm.	gm.
Arginine	1.2	4.7	4.7	7.2	4.3	3.9	3.5
Histidine	2.4	1.6	2.0	2.1	2.5	2.2	2.3
Lysine	6.0	4.6	5.2	8.1	7.5	2.0	2.8
Tyrosine		3.9	3.9	3.1	5.4	3.8	4.4
Tryptophane	1.2	0.9	1.1	1.2	1.6	1.0	1.3
Phenylalanine	4.2	4.2	4.7	4.5	5.7	5.5	5.1
Cystine + Methionine	3.6	3.7	4.1	4.2	4.0	4.2	4.2
Threonine	3.6	3.2	3.6	4.3	4.6	2.7	2.8
Leucine	5.4	9.6	12.6	12.1	16.2	12.0	11.2
Isoleucine	3.0	3.1	3.7	3.4	4.4	3.7	3.3
Valine	4.2	3.2	3.9	3.4	5.5		3.1

* Enriched bread contained 6 per cent milk solids and high vitamin yeast.

TABLE III

Percentage of Optimal Daily Requirement of Each of the Essential
Amino Acids Supplied by 100 gm. of Protein from Meat, Milk,
White Flour, "Vitamin Enriched" Bread, Corn, and Soybeans

AMINO ACID	AVERAGE REQUIRE- MENTS	SUPPLIED BY 100 GM. OF PROTEIN FROM					
	Calcu- lated	Meat	Milk	White Flour	"Enriched" Bread	Corn	Soybeans
	gm.	%	%	%	%	%	%
Arginine	3.5	210	125	110	110	115	165
Histidine	2.0	105	125	110	113	120	115
Lysine	5.2	145	140	40	55	40	105
Tyrosine	3.9	80	140	100	115	155	105
Tryptophane	1.1	110	175	90	120	55	145
Phenylalanine	4.4	100	130	125	115	105	125
Cystine + Methionine	3.8	110	105	110	110	130	80
Threonine	3.5	125	135	80	80	105	120
Leucine	9.1	135	180	130	120	240	90
Isoleucine	3.3	105	135	110	100	115	125
Valine	3.8	90	145		80	120	115

Enriched bread contained 6 per cent milk solids and high vitamin yeast.

BIBLIOGRAPHY

1. Abderhalden, E.: Hydrolyse des krystallisirten Oxyhämoglobins aus Pferdeblut. Zeit. physiol. Chem. 1902–03, **37**, 484–494.
2. Abderhalden, E.: Hydrolyse des krystallisirten Serum-albumins aus Pferdeblut. Zeit. physiol. Chem. 1902–03, **37**, 495–498.
3. Abderhalden, E.: Hydrolyse des Edestins. Zeit. physiol. Chem. 1902–03, **37**, 499–505.
4. Abderhalden, E. and Schittenhelm, A.: Die Abbauprodukte des Elastins. Zeit. physiol. Chem. 1904, **41**, 293–298.
5. Abderhalden, E.: Abbau und Aufbau der Eiweisskörper im Tierschen Organismus. Zeit. physiol. Chem. 1905, **44**, 17–52.
6. Abderhalden, E. and Rostoski, O.: Die Monoaminosäuren des "Edestins" aus Baumwollsamen und dessen Verhalten gegen Magensaft. Zeit. physiol. Chem. 1905, **44**, 265–275.
7. Abderhalden, E. and Samuely, F.: Die Zusammensetzung des "Gliadins" des Weizenmehles. Zeit. physiol. Chem. 1905, **44**, 276–283.
8. Abderhalden, E. and Reinhold, B.: Die Monoaminosäuren des "Edestins" aus Sonnenblumensamen und dessen Verhalten gegen Pankreassaft. Zeit. physiol. Chem. 1905, **44**, 284–293.
9. Abderhalden, E. and Wells, H. G.: Die Monoaminosäuren des Keratins aus Pferdehaaren. Zeit. physiol. Chem. 1905, **46**, 31–39.
10. Abderhalden, E. and LeCount, E. R.: Die Monoaminosäuren des Keratins aus Gänsefedern. Zeit. physiol. Chem. 1905, **46**, 40–46.
11. Abderhalden, E. and Rostoski, O.: Beitrag zur Kenntnis des Bence-Jonesschen Eiweisskörpers. Zeit. physiol. Chem. 1905, **46**, 125–135.
12. Abderhalden, E. and Strauss, E.: Die Spaltprodukte des Spongins mit Säuren. Zeit. physiol. Chem. 1906, **48**, 49–53.
13. Abderhalden, E. and Hunter, A.: Hydrolyse des im Eigelb des Hühnereies enthaltenen Proteins ("Vitellin"). Zeit. physiol. Chem. 1906, **48**, 505–512.
14. Abderhalden, E. and Ebstein, E.: Die Monoaminosäuren der Schalenhaut des Hühnereies. Zeit. physiol. Chem. 1906, **48**, 530–534.
15. Abderhalden, E. and Kempe, M.: Beitrag zur Kenntnis des Tryptophans und einiger seiner Derivate. Zeit. physiol. Chem. 1907, **52**, 207–218.
16. Abderhalden, E. and Voitinovici, A.: Hydrolyse des Keratins aus Horn und aus Wolle. Zeit. physiol. Chem. 1907, **52**, 348–367.
17. Abderhalden, E. and Langstein, L.: Vergleichende Untersuchung über die Zusammensetzung des Caseins aus Frauen- und Kuhmilch. Zeit. physiol. Chem. 1910, **66**, 8–12.
18. Abderhalden, E. and Weil, A.: Über die bei der Isolierung der Monoaminosäuren mit Hilfe des Estermethode entstehenden Verluste. I. Zeit. physiol. Chem. 1911, **74**, 445–471.

311

19. Abderhalden, E. and Weil, A.: Über die bei der Isolierung der Monoaminosäuren mit Hilfe des Estermethode entstehenden Verluste. II. Zeit. physiol. Chem. 1912, **77**, 59–74.

20. Abderhalden, E.: Beitrage zur Kenntnis der Zusammensetzung des Seidenfibroins und seiner Struktur. Zeit. physiol. Chem. 1922, **120**, 207–213.

21. Abderhalden, E., Fleischmann, R. and Irion, W.: Fortgesetzte Studien über das Problem der spezifischen Einstellung der Polypeptide spaltenden Fermentkomplexe. Versuche mit Histidin als Baustein enthaltenden Polypeptiden. Fermentforsch. 1928–29, **10**, 446–454.

22. Abderhalden, E. and Neumann, A.: Verwendung des Orthophthaldialdehyde-Reagenses nach Walther Zimmermann zum Nachweis kleiner Mengen von Glykokoll und zur Bestimmung seiner stellung in Polypeptiden. Zeit. physiol. Chem. 1936, **238**, 177–182.

23. Abderhalden, E. and Siebel, H.: Beitrag zum Problem der Abhängigkeit der Zusammensetzung von Eiweisstoffen des Blutplasmas bzw. -serums von der Art der aufgenommenen Nahrung. Zeit. physiol. Chem. 1936, **238**, 169–176.

24. Abderhalden, E., Baertich, E. and Zeisecke, W.: Über den Gehalt von aus Kabeljau gewonnenem Muskeleiweiss an Aminosäuren. Zeit. physiol. Chem. 1936, **240**, 152–162.

25. Ågren, G.: Über die pharmakodynamischen Wirkungen und chemischen Eigenschaften des Secretins. Skand. Arch. f. Physiol. 1934, **69**, 1–78.

26. Ågren, G.: On the Properties of Secretin. J. Physiol. 1939, **94**, 553–559.

27. Albanese, A. A.: An Electrolytic Method for the Determination of the Basic Amino Acids in Proteins. J. Biol. Chem. 1940, **134**, 467–482.

28. Albanese, A. A. and Frankston, J. E.: A New Color Test for Tryptophane in Protein Hydrolysates. J. Biol. Chem. 1942, **144**, 563–564.

29. Almquist, H. J., Mecchi, E., Kratzer, F. H. and Grau, C, R.: Soybean Protein as a Source of Amino Acids for the Chick. J. Nutr. 942, **24**, 385–392

30. Alving, A. S. and Mirsky, A. E.: The Nature of Plasma and Urinary Proteins in Nephrosis. J. Clin. Invest. 1936, **15**, 215–220.

31. Andrews, J. C. and Andrews, K. C.: Substances which Inhibit Color Development in the Sullivan Method for Cystine. J. Biol. Chem. 1937, **118**, 555–567.

32. Argiris, A.: Zur Kenntnis des Neurokeratins. Zeit. physiol. Chem. 1907–08, **54**, 86–94.

33. Arhimo, A. A.: Determination of Small Amounts of Aspartic Acid with the Malic Acid Method of Pucher. Suomen Kemistilehti, 1939, **12B**, 6.

34. Arhimo, A. A. and Laine, T.: Determination of Glutamic Acid. Suomen Kemistilehti, 1939, **12B**, 18.

35. Arnold, V.: Eine Farbenreaktion von Eiweisskörpern mit Nitro-prussidnatrium. Zeit. physiol. Chem. 1910–11, **70**, 300–309.

36. Arnow, L. E.: Colorimetric Determination of the Components of 3,4-Dihydroxyphenylalanine-Tyrosine Mixtures. J. Biol. Chem. 1937, **118**, 531–537.

37. Arnow, L. E., Burns, J. and Bernhart, F. W.: Phenylalanine Content of Hen's Egg Albumin. Proc. Soc. Exp. Biol. and Med. 1939, **41**, 499–500.

38. Ayre, C. A.: The Lysine Content of Feeding Stuffs. Biochem. J. 1938, **32**, 1152–1156.

39. Baernstein, H. D.: The Gasometric Determination of Cysteine and Cystine. J. Biol. Chem. 1930, **89**, 125–131.

40. Baernstein, H. D.: The Determination of Methionine in Proteins. J. Biol. Chem. 1932, **97**, 663–668.

41. Baernstein, H. D.: The Sulfur Distribution in Proteins. J. Biol. Chem. 1932, **97**, 669–674.

42. Baernstein, H. D.: A New Method for the Determination of Methionine in Proteins. J. Biol. Chem. 1936, **115**, 25–32.

42A. Baernstein, H. D.: The Sulfur Distribution in Proteins. II. The Combined Methods for the Determination of Cystine, Methionine, and Sulfates in Hydriodic Acid Digests. J. Biol. Chem. 1936, **115**, 33–36.

43. Bailey, K.: The Sulphur Distribution of Proteins. Biochem. J. 1937, **31**, 1396–1405.

44. Bailey, K.: Composition of the Myosins and Myogen of Skeletal Muscle. Biochem. J. 1937, **31**, 1406–1413.

45. Bálint, P.: Stufenphotometrische Tyrosin-, Tryptophan-, und Cystin-Bestimmung (Auf Grund des kolorimetrischen Verfahrens von Folin und Marenzi). Biochem. Zeit. 1938, **299**, 133–136.

46. Bálint, P. and Bálint, M.: Über die chemische Zusammensetzung der menschlichen Bluteiweisskörper. Biochem. Zeit. 1940, **305**, 310–322.

47. Bálint, P. and Bálint, M.: Chemical Composition of Human Blood Proteins. Biochem. Zeit. 1941, **308**, 82–88, from Chem. Abs. 1943, **37**, 147.

48. Balls, A. K.: A Crystalline Sulfur-Protein from Wheat. J. Washinton Acad. Sci. 1942, **32**, 132–137.

49. Barker, S. B. and Summerson, W. H.: The Colorimetric Determination of Lactic Acid in Biological Material. J. Biol. Chem. 1941, **138**, 535–554.

50. Barger, G. and Coyne, F. P.: The Amino Acid Methionine; Constitution and Synthesis. Biochem. J. 1928, **22**, 1417–1425.

51. Barnes, H. and Peters, R. A.: The Use of Pure Phosphotungstic Acids in the Precipitation of Bases. I. Method of Checking the Purity of 1:24 Phosphotungstic Acid. II. The Influence of Hydrion Concentration and Some Other Factors on the Precipitation of Bases. Biochem. J. 1932, **26**, 2203–2219.

52. Barritt, J.: The Distribution and Origin of Sulfur in Wool. I. Methionine in Wool. Biochem. J. 1934, **28,** 1–5.

53. Bastian, M. A.: Dosage de la Proline dans un Hydrolysat de Gélatine. Bull. Soc. Chim. biol. 1937, **19,** 1299–1301.

54. Bates, R. W.: A Rapid Method for Quantitative Determination of Tryptophane. Proc. Amer. Soc. Biol. Chem.; J. Biol. Chem. 1937, **119,** VII.

55. Beach, E. F., Erickson, B. N., Bernstein, S. S., Williams, H. H. and Macy, I. G.: The Amino Acid Composition of Erythrocyte Post-hemolytic Residue of Five Mammalian Species. J. Biol. Chem. 1939, **128,** 339–346.

56. Beach, E. F., Bernstein, S. S., Hummel, F. C., Williams, H. H. and Macy, I. G.: Total Sulfur, Cystine, and Methionine Content of Blood Globins of Five Mammalian Species. J. Biol. Chem. 1939, **130,** 115–119.

57. Beach, E. F., Bernstein, S. S., Hoffman, O. D., Teague, D. M. and Macy, I. G.: Distribution of Nitrogen and Protein Amino Acids in Human and in Cow's Milk. J. Biol. Chem. 1941, **139,** 57–63.

58. Beach, E. F. and Teague, D. M.: A Gravimetric Method for the Determination of Methionine. J. Biol. Chem. 1942, **142,** 277–284.

59. Beach, E. F., Munks, B. and Robinson, A.: The Amino Acid Composition of Animal Tissue Protein. J. Biol. Chem. 1943, **148,** 431–439.

60. Beadle, G. W. and Tatum, E. L.: Genetic Control of Biochemical Reactions in Neurospora. Proc. Nat. Acad. Sci. 1941, **27,** 499–506, and private communication.

61. Benedict, S. R.: A Note on the Preparation of Glyoxylic Acid as a Reagent. J. Biol. Chem. 1909, **6,** 51–52.

62. Benedict, S. R.; A Note on the Purification of Picric Acid for Creatinine Determinations. J. Biol. Chem. 1929, **82,** 1–3.

63. Bennett, M. A.: Metabolism of Sulfur. VII. A Quantitative Study of the Replaceability of *l*-Cystine by various Sulfur-Containing Amino-Acids in the Diet of the Albino Rat. Biochem. J. 1939, **33,** 885–892.

64. Bergmann, M. and Fox, S. W.: Complex Salts of Amino Acids and Peptides. I. Metal Complex Salts of Glycine and Their Specificity. J. Biol. Chem. 1935, **109,** 317–324.

65. Bergmann, M.: Complex Salts of Amino Acids and Peptides. II. Determination of *l*-Proline with the Aid of Rhodanilic Acid. The Structure of Gelatin. J. Biol. Chem. 1935, **110,** 471–479.

66. Bergmann, M. and Niemann, C.: On Blood Fibrin. A Contribution to the Problem of Protein Structure. J. Biol. Chem. 1936, **115,** 77–85.

67. Bergmann, M. and Niemann, C.; On the Structure of Proteins: Cattle Hemoglobin, Egg Albumin, Cattle Fibrin, and Gelatin. J. Biol. Chem. 1937, **118,** 301–314.

68. Bergmann, M.: Complex Salts of Amino Acids and Peptides. III.

Salts of Dioxalatopyridinochromiato Acid (Dioxypyridic Acid) and Dioxalatodianilinochromiato Acid (Dioxanilic Acid). J. Biol. Chem. 1937–38, 122, 569–576.

69. Bergmann, M. and Niemann, C.; On the Structure of Silk Fibroin. J. Biol. Chem. 1937–38, 122, 577–596.

70. Bergmann, M. and Stein, W. H.: A New Principle for the Determination of Amino Acids, and its Application to Collagen and Gelatin. J. Biol. Chem. 1939, 128, 217–232.

71. Bergmann, M. and Stein, W. H.: Naphthalene-β-sulfonic Acid as a Reagent for Amino Acids. J. Biol. Chem. 1939, 129, 609–618.

72. Bernhart, F. W.: Microdetermination of the Tyrosine Content of Protein Hydrolysates. Proc. Amer. Soc. Biol. Chem.; J. Biol. Chem. 1938, 123, X.

73. Bernhart, F. W.: Molecular Weight of Egg Albumin. J. Biol. Chem. 1940, 132, 189–193.

74. Biale, J. B. and Weiss, F. T.: Identification of Acetaldehyde in the Steam Distillate of the Peel of Citrus Fruits. J. Amer. Chem. Soc. 1939, 61, 635–636.

75. Birkofer, L. and Taurins, A.: Quantitative Bestimmung der schwefelhaltigen Aminosäuren in verschiedenen Globinen. Zeit. physiol. Chem. 1940, 265, 94–101.

76. Blau, N. F.: The Determination of Thyroxine in Thyroid Substance. J. Biol. Chem. 1935, 110, 351–363.

77. Block, R. J. and Vickery, H. B.: The Basic Amino Acids of Proteins. A Chemical Relationship between Various Keratins. J. Biol. Chem. 1931, 93, 113–117.

78. Block, R. J.: The Basic Amino Acids from Neurokeratin: Is Neurokeratin a True Keratin? J. Biol. Chem. 1931–32, 94, 647–651.

79. Block, R. J.: Basic Amino Acids of Human Skin. Proc. Soc. Exp. Biol. and Med. 1934–35, 32, 1574–1575.

80. Block, R. J.: The Basic Amino Acids of Keratins. The Basic Amino Acid Content of Human Finger Nails and Cattle Horn. J. Biol. Chem. 1934, 104, 339–341.

81. Block, R. J.: The Basic Amino Acids of Serum Proteins. II. The Effect of Heating to Fifty-Eight Degrees. J. Biol. Chem. 1934, 104, 343–346.

82. Block, R. J., Darrow, D. C. and Cary, M. K.: The Basic Amino Acids of Serum Proteins. III. A Chemical Relationship between Serum Proteins of Various Origins. J. Biol. Chem. 1934, 104, 347–350.

83. Block, R. J.: The Basic Amino Acids of Serum Proteins (Orosins). IV. A Chemical Relationship between Various Avian Orosins. A Note on Some Proteins of the Egg. J. Biol. Chem. 1934, 105, 455–461.

84. Block, R. J.: The Basic Amino Acids of Three Crystalline Mammalian Hemoglobins. Further Evidence for a Basic Amino Acid "Anlage" of Tissue Proteins. J. Biol. Chem. 1934, 105, 663–666.

85. Block, R. J., Jones, D. B. and Gersdorff, C. E. F.: The Effect of Dry Heat and Dilute Alkali on the Lysine Content of Casein. J. Biol. Chem. 1934, **105**, 667–668.

86. Block, R. J.: The Determination of the Basic Amino Acids in Small Quantities of Proteins by the Silver Precipitation Method. J. Biol. Chem. 1934, **106**, 457–462.

87. Block, R. J.: On the Nature and Origin of Proteins. Yale J. Biol. and Med. 1935, **7**, 235–252.

88. Block, R. J.: The Estimation of Histidine. Proc. Soc. Exp. Biol. and Med. 1937–38, **37**, 580–582.

89. Block, R. J.: Chemical Studies on the Neuroproteins. I. The Amino Acid Composition of Various Mammalian Brain Proteins. J. Biol. Chem. 1937, **119**, 765–768.

90. Block, R. J.: Chemical Studies on the Neuroproteins. II. The Effect of Age on the Amino Acid Composition of Human and Mammalian Brain Proteins. J. Biol. Chem. 1937, **120**, 467–470.

91. Block, R. J. and Horwitt, M. K.: The Basic Amino Acids of Keratins. A Note on the Basic Amino Acid Content of Porcupine Quills and Echidna Spines. J. Biol. Chem. 1937, **121**, 99–100.

92. Block, R. J.: Chemical Studies on the Neuroproteins. III. An Indication for Sex Differences in the Amino Acid Composition of Primate Brain Proteins. J. Biol. Chem. 1937, **121**, 411–416.

93. Block, R. J.: Chemical Studies on the Neuroproteins. IV. On the Nature of the Proteins of the Ectoderm: Eukeratins and Pseudokeratins. J. Biol. Chem. 1937, **121**, 761–770.

94. Block, R. J. Comparative Biochemistry of the Proteins. Cold Spring Harbor Symposia 1938, **6**, 79–90.

95. Block, R. J.: The Determination of the Amino Acids. Burgess Publishing Co., Minneapolis, Minn. 1938, 85 pages.

96. Block, R. J. and Bolling, D.: The Amino Acid Composition of Keratins. The Composition of Gorgonin, Spongin, Turtle Scutes, and other Keratins. J. Biol. Chem. 1939, **127**, 685–693.

97. Block, R. J.: The Amino Acid Composition of Hair, Wool, Horn, and other Eukeratins. J. Biol. Chem. 1939, **128**, 181–186.

98. Block, R. J. and Bolling, D.; Chemical and Metabolic Studies on Phenylalanine. I. The Nitration of Phenylalanine. J. Biol. Chem. 1939, **129**, 1–12.

99. Block, R. J. and Bolling, D.: Determination of Threonine. Proc. Soc. Exp. Biol. and Med. 1939, **40**, 710.

100. Block, R. J. and Bolling, D.; The Microestimation of Threonine. J. Biol. Chem. 1939, **130**, 365–374.

101. Block, R. J.: The Estimation of Histidine. J. Biol. Chem. 1940, **133**, 67–69.

102. Block, R. J.: Basic Amino Acid Content of Human Serum Proteins. The Influence of the Ingestion of Arginine on the Composition of the Serum Proteins. J. Biol. Chem. 1940, **133**, 71–74.

103. Block, R. J., Bolling, D. and Webb, M.: The Microestimation of

Alanine and Threonine. Proc. Amer. Soc. Biol. Chem.; J. Biol. Chem. 1940, **133**, XIV.

104. Block, R. J. and Bolling, D.: The Determination of the Amino Acids. Burgess Publishing Co., Minneapolis, Minn. 1940, 58 pages.

105. Block, R. J., Jervis, G. A., Bolling, D. and Webb, M.: Chemical and Metabolic Studies on Phenylalanine. III. The Amino Acid Content of Tissue Proteins of Normal and Phenylpyruvic Oligophrenic Individuals. A Note on the Estimation of Phenylalanine. J. Biol. Chem. 1940, **134**, 567–572.

106. Block, R. J., Bolling, D. and Kondritzer, A. A.: Microestimation of Leucine, Isoleucine, and Valine. Proc. Soc. Exp. Biol. and Med. 1940, **45**, 289–292.

107. Block, R. J.: A New Method for the Separation of the Basic Amino Acids from Protein Hydrolysates. Proc. Soc. Exp. Biol. and Med. 1942, **51**, 252–253.

108. Block, R. J.: The Essential Amino Acid Requirements of Man. Yale J. Biol. and Med. 1943, **15**, 722–728.

108A. Block, R. J. and Bolling, D.: National Opportunities with Amino Acids. J. Amer. Diet. Assoc. 1944, **20**, 69–76.

109. Block, W. D. and Lewis, H. B.: The Amino Acid Content of Cow and Chimpanzee Hair. J. Biol. Chem. 1938, **125**, 561–570.

110. Block, W. D. and Murrill, W. A.: Comparative Amino Acid Content of Serum Proteins in Normal Humans and in Patients with Rheumatoid Arthritis. Proc. Soc. Exp. Biol. and Med. 1941, **47**, 374–376.

111. Blumenthal, D. and Clarke, H. T.: Unrecognized Forms of Sulfur in Proteins. J. Biol. Chem. 1935, **110**, 343–349.

112. Bolling, D. and Block, R. J.: Amino Acids Yielded by β-Lactoglobulin. Archiv. of Biochem., 1943, **2**, 93–95.

113. Bolling, D. Unpublished experiments.

114. Bonot, A. and Cahn, T.: Dosage de l'Arginine par la méthode de Jansen modifée. Bull. Soc. Chim. biol. 1927, **9**, 1001–1006.

115. Borchers, R., Totter, J. R. and Berg, C. P.: Threonine Deficiency in Hydrolysates of Zein Prepared by Autoclaving. J. Biol. Chem. 1942, **142**, 697–704.

116. Bovarnick, M.: The Formation of Extracellular $d(-)$Glutamic Acid Polypeptide by Bacillus Subtilis. J. Biol. Chem. 1942, **145**, 415–424.

117. Boyd, W. J.: Note on the Determination of Tryptophane by Means of p-Dimethylaminobenzaldehyde. Biochem. J. 1929, **23**, 78–82.

118. Boyd, W. J.: The Isolation of Amino-Acids in the Form of the Corresponding Carbamido-Acids and Hydantoins. I. The Derivatives of the Mono-Amino-Mono-Carboxylic Acids. Biochem. J. 1933, **27**, 1838–1848.

119. Boyd, M. J. and Logan, M. A.: Colorimetric Determination of Serine. J. Biol. Chem. 1942, **146**, 279–287.

120. Boyd, M. J.: Colorimetric Determination of Serine. Private Communication.

121. Brand, E., Block, R. J., Kassell, B. and Cahill, G. F.: Cystinuria

V. The Metabolism of Casein and Lactalbumin. J. Biol. Chem. 1937, **119**, 669–680.

122. Brand, E., Kassell, B. and Heidelberger, M.: On the Structure of Thyroglobulin. Proc. Amer. Soc. Biol. Chem.; J. Biol. Chem. 1939, **128**, XI.

123. Brand, E. and Kassell, B.: The Photometric Determination of Tryptophane, Tyrosine, Diiodotyrosine, and Thyroxine. J. Biol. Chem. 1939, **131**, 489–502.

124. Brand, E. and Kassell, B.: The Determination of Reducing Groups with Porphyrindin, with Special Reference to Egg Albumin. J. Biol. Chem. 1940, **133**, 437–444.

125. Brand, E. and Kassell, B.: Determination of Certain Amino Acids in Chymotrypsinogen, and its Molecular Weight. J. Gen. Physiol. 1941–42, **25**, 167–176.

126. Brand, E. and Kassell, B.: Absence of Methionine in Crystalline Horse Serum Albumin. J. Biol. Chem. 1941, **141**, 999–1000.

127. Brand, E. and Kassell, B.: Photometric Determination of Arginine. J. Biol. Chem. 1942, **145**, 359–364.

128. Brand, E. and Kassel, B.: Analysis and Minimum Molecular Weight of β-Lactoglobulin. J. Biol. Chem. 1942, **145**, 365–378.

129. Brazier, M. A. B.: A New Method for the Separation of the Products of Protein Hydrolysis. Biochem. J. 1930, **24**, 1188–1198.

130. Brdicka, R.: Polarographic Studies in the Dropping Mercury Kathode. XXXI. A New Test for Proteins in the Presence of Cobalt Salts in Ammoniacal Solutions of Ammonium Chloride. Collect. Czechoslov. Chem. Communicat. 1933, **5**, 112–128.

131. Brdicka, R.: Polarographische Mikrobestimmung von Cystin und Cystein in Hydrolysaten von einigen Proteinen. Mikrochemie 1934, **15**, 167–180.

132. Brice, A. T.: The Tryptophane Content of Blood Serum. A New Technique. J. Lab. Clin. Med. 1933–34, **19**, 1113–1115.

133. Brown, W. L.: The Threonine, Serine, Cystine, and Methionine Content of Peanut Proteins. J. Biol. Chem. 1942, **142**, 299–301.

134. Brown, W. L. Private Communication, April, 1942.

135. Brummer, P.: The Determination of Tryptophane and the Tryptophane Content of Serum Proteins. Chem. Abs. 1942, **36**, 6566.

136. Bushill, J. H., Lampitt, L. H. and Baker, L. C.: The Determination of Cystine. Use of the Zeiss Photometer. Biochem. J. 1934, **28**, 1293–1304.

137. Bussit, J.: Recherches analytiques sur l'arginine et l'histidine. Herrmann et Cie. Paris, 1934–35, 113 pages.

137A. Callan, T. P. and Toennies, G.: Determination of Sulfur in Organic Compounds. Ind. and Eng. Chem.; Anal. Ed. 1941, **13**, 450–455.

138. Calvery, H. O.: Basic Amino Acids. The Estimation of the Basic Amino Acids in Small Amounts of Casein and Edestin by the Modified Method of Vickery and Leavenworth and other Methods. J. Biol. Chem. 1929, **83**, 631–648.

139. Calvery, H. O.: Studies on Crystallized Egg Albumin. J. Biol. Chem. 1931-32, **94**, 613-634.
140. Calvery, H. O. and White, A.: Vitellin of Hen's Egg. J. Biol. Chem. 1931-32, **94**, 635-639.
141. Calvery, H. O.: Some Chemical Investigations of Embryonic Metabolism. VI. Studies of Some of the Amino Acids of the Yolk, White, Embryo, and Shell Membranes during Development of the Hen's Egg. J. Biol. Chem. 1932, **95**, 297-300.
142. Calvery, H. O.: Some Analyses of Egg-Shell Keratin. J. Biol. Chem. 1933, **100**, 183-186.
143. Calvery, H. O. and Freyberg, R. H.: Some Analyses of Samples of Bence-Jones Protein. J. Biol. Chem. 1935, **109**, 739-743.
144. Calvery, H. O.: Analysis of Type II Pneumococcus Specific Precipitate. Proc. Soc. Exp. Biol. and Med. 1934-35, **32**, 1099-1100.
145. Calvery, H. O.: Analysis of Type I Pneumococcus Specific Precipitate. J. Biol. Chem. 1935-36, **112**, 167-169.
146. Calvery, H. O., Herriott, R. M. and Northrup, J. H.: The Determination of Some Amino Acids in Crystalline Pepsin. J. Biol. Chem. 1936, **113**, 11-14.
147. Cannan, R. K., Palmer, A. H. and Kibrick, A. C.: The Hydrogen Ion Dissociation Curve of β-Lactoglobulin. J. Biol. Chem. 1942, **142**, 803-822.
148. Cannan, R. K. Private Communication, Dec. 1942.
149. Cary, C. A.: The Use of the Hopkins-Cole Glyoxylic Acid Reagent for the Quantitative Determination of Free Tryptophane in Blood. J. Biol. Chem. 1926, **67**, XXXIX.
150. Catch, J. R., Cook, A. H. and Heilbron, I. M.: Purification and Chemistry of Penicillin. Nature 1942, **150**, 633-634.
151. Cavett, J. W.: A Modification of the Van Slyke Nitrogen Distribution Method. J. Biol. Chem. 1932, **95**, 335-343.
152. Cavett, J. W.: Thyroglobulin Studies. II. The Van Slyke Nitrogen Distribution and Tyrosine and Tryptophane Analyses for Normal and Goitrous Human Thyroglobulin. J. Biol. Chem. 1936, **114**, 65-73.
153. Cavett, J. W. Private Communication, March, 1942.
154. Chaudhuri, T. C.: A New Synthesis of Phthaldehydes. J. Amer. Chem. Soc. 1942, **64**, 315.
155. Cheldelin, V. H. and Williams, R. J.: Adsorption of Organic Compounds. 1. Adsorption of Ampholytes on an Activated Charcoal. J. Amer. Chem. Soc. 1943, **64**, 1513-1516.
156. Cherbuliez, E., Plattner, P. and Ariel, S.: Sur le dosage des acides aminés formés par l'hydrolyse des protides. III. Application du procédé d'éthérification et d'acétylation aux produits d'hydrolyse de protides. Helv. Chem. acta 1930, **13**, 1390-1402.
157. Chibnall, A. C., Rees, M. W., Williams, E. F. and Boyland, E.: The Glutamic Acid of Normal and Malignant Tissue Proteins. Biochem. J. 1940, **34**, 285-300.

157A. Chibnall, A. C., Rees, M. W. and Williams, E. F.: The Total Nitrogen Content of Egg Albumin and other Proteins. Biochem. J. 1943, **37**, 354–359.

158. Chibnall, A. C., cf. Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F.: Critique of the Foreman Method for the Estimation of the Dicarboxylic Acids in Protein Hydrolysates. Biochem. J. 1943, **37**, 360–372.

159. Chibnall, A. C., cf. Rees, M. W. and Williams, E. F.: The Dicarboxylic and Basic Amino Acids in Edestin, Egg Albumin, and β-Lactoglobulin. Biochem. J. 1943, **37**, 372–388.

160. Chibnall, A. C.: Amino Acid Analysis and the Structure of Protein. The Bakerian Lecture, 1942.

161. Christensen, B. E., West, E. S. and Dimick, K. P.: A Simple Apparatus and Procedure for Determination of Amino Acids by the Ninhydrin Reaction. J. Biol. Chem. 1941, **137**, 735–738.

162. Christensen, H. N., Edwards, R. R. and Piersma, H. D.: The Composition of Gramicidin and Tyrocidine. J. Biol. Chem. 1941, **141**, 187–195.

163. Clancy, V. J.: The Constitution of Sponges. I. The Common Bath Sponge, *Hippospongia Equina*. Biochem. J. 1926–27, **20**, 1186–1189.

164. Clarke, H. T. and Inouye, J. M.: Some Observations on the Action of Alkali upon Cystine and Cysteine. J. Biol. Chem. 1930, **89**, 399–419.

165. Clarke, H. T.: The Action of Sulfite upon Cystine. J. Biol. Chem. 1932, **97**, 235–248.

166. Clay, R. C., Cook, K. and Routh, J. I.: Studies in the Composition of Human Hair. J. Amer. Chem. Soc. 1940, **62**, 2709–2710.

167. Cohen, P. P.: Micro-determination of Glutamic Acid. Biochem. J. 1939, **33**, 551–558.

168. Conrad, R. M. and Berg, C. P.: The Optical Inversion of *d*-Histidine in the Animal Body. J. Biol. Chem. 1937, **117**, 351–363.

169. Conway, E. J. and Byrne, A.: An Absorption Apparatus for the Micro-Determination of Certain Volatile Substances. I. The Micro-Determination of Ammonia. Biochem. J. 1933, **27**, 419–429.

170. Conway, E. J. and O'Malley, E.: Microdiffusion Methods. Ammonia and Urea Using Buffered Adsorbents (Revised Methods for Ranges Greater than 10 ug. N). Biochem. J. 1942, **36**, 655–661.

171. Cook, C. A. and Smith, A. H.: The Determination of Isopropyl Alcohol in the Presence of Acetone in the Urine. J. Biol. Chem. 1929–30, **85**, 251–260.

172. Cox, C. J., King, H. and Berg, C. P.: The Preparation of Lysine, Histidine, and Arginine from Hydrolyzed Blood Corpuscle Paste by Electrical Transport. J. Biol. Chem. 1929, **81**, 755–764.

173. Criegee, R., Kraft, L. and Rank, B.: Die Glykolspaltung, ihr Mechanismus und ihre Anwendung auf chemische Probleme. Liebig's Ann. 1933. **507**, 159–197.

174. Croft, H.: On a New Oxalate of Chromium and Potash. The London, Edinburgh, and Dublin Philosophical Mag. and J. of Science. 1842. **21**, 197–200.

175. Csonka, F. A. and Jones, D. B.: Studies on Glutelins. I. The α and β-Glutelins of Wheat (*Triticum Vulgare*). J. Biol. Chem. 1927, **73**, 321–329.

176. Csonka, F. A. and Jones, D. B.: Studies on Glutelins. V. The Glutelins of Rye (*Secale Cereale*) and of Barley (*Hordeum Vulgare*). J. Biol. Chem. 1929, **82**, 17–21.

177. Csonka, F. A.: Studies on Glutelins. VII. Cystine, Tryptophane, and Tyrosine Content of Glutelins. J. Biol. Chem. 1932, **97**, 281–286.

178. Csonka, F. A.: Proteins of Yeast (*Saccharomyces Cerevisiae*). J. Biol. Chem. 1935, **109**, 703–715.

179. Csonka, F. A.: Amino Acids in Staple Foods. I. Wheat (*Triticum Vulgare*). J. Biol. Chem. 1937, **118**, 147–153.

180. Csonka, F. A.: Amino Acids in Staple Foods. II. The Effect of Milling Wheat on the Distribution of Amino Acids. Cereal Chem. 1937b, **14**, 397–399.

181. Csonka, F. A.: Amino Acids in the Corn Kernel. J. Agri. Research 1939, **59**, 765–768.

182. Csonka, F. A.: Amino Acids in Oats and Oat Milling Products, Including Rolled Oats. Cereal Chem. 1941, **18**, 523–529.

183. Dakin, H. D.: On Amino Acids. Biochem. J. 1918, **12**, 290–317.

184. Dakin, H. D.: On Amino Acids. Part II. Hydroxyglutamic Acid. Biochem. J. 1919, **13**, 398–429.

185. Dakin, H. D.: Amino-Acids of Gelatin. J. Biol. Chem. 1920, **44**, 499–52 .

186. Dakin, H. D.: Die Aminosäuren des Zein. Zeit. physiol. Chem. 1923, **130**, 159–168.

187. Dakin, H. D.: A Note on the Presence of Valine in Zein. J. Biol. Chem. 1924, **61**, 137–138.

188. Dakin, H. D. and West, R.: Observations on the Chemical Nature of a Hematopoietic Substance Occurring in Liver. J. Biol. Chem. 1935, **109**, 489–522

189. Dakin, H. D.: Reinecke Salt. Organic Syntheses 1935, **15**, 74–76.

190. Dakin, H. D.: On Methylaspartic Acids and Their Methylation. J. Biol. Chem. 1941, **141**, 945–950.

191. Dakin, H. D.: On Lysine and Ornithine. J. Biol. Chem. 1942, **146**, 237–240.

192. von Descö, D.: Beitrag zur kolorimetrischen Bestimmung des Tyrosins und des Tryptophans nach Folin und Marenzi. Biochem. Zeit. 1934, **271**, 142–151.

193. von Descö, D., Lamoth, E. and Fodor, L.: Über den Tyrosin- und Tryptophangehalt des Globulins und Albumins beim neugeborenen Kalbe und beim Muttertier. Biochem. Zeit. 1934, **271**, 381–384.

194. Desneulle, P.: Sur le dosage colorimétrique de l'alanine et de la somme sérine et acide aspartique dans les protéines. Application à la protéine du ferment jaune. Enzymologia 1938, **5**, 37–43.

195. Devine, J.: An Analysis of Bence-Jones Protein. Biochem. J. 1941, **35**, 433–445

196. Dische, Z.: Über einige neue charakteristische Farbreaktionen der Thymonukleinsäure und eine Mikromethode zur Bestimmung der selben in tierschen Organen mit Hilfe dieser Reaktionen. Mikrochemie 1930, **8**, 4–32.

197. Doherty, D. G., Stein, W. H. and Bergmann, M.: Aromatic Sulfonic Acids as Reagents for Amino Acids. J. Biol. Chem. 1940, **135**, 487–496.

198. Dubnoff, J. W. and Borsook, H.: A Micromethod for the Determination of Glycocyamine in Biological Fluids and Tissue Extracts. J. Biol. Chem. 1941, **138**, 381–388.

199. Dubnoff, J. W.: A Micromethod for the Determination of Arginine. J. Biol. Chem. 1941, **141**, 711–716.

200. Dumazert, C. and Poggi, R.: Sur le microdosage colorimétrique de l'arginine et de divers dérivés guandiques monosubstitués. Application aux·hydrolysates protéiques. Bull. Soc. Chim. biol. 1939, **21**, 1381–1388.

202. Eckstein, H. C.: The Distribution of Some of the More Important Amino Acids in the Globulin of the Thyroid Gland. J. Biol. Chem. 1926, **67**, 601–607.

203. Eckstein, H. C.: Amino Acids in Human Skin. Proc. Soc. Exp. Biol. and Med. 1934–35, **32**, 1573–1574.

204. Edlbacher, S., Baur, H., Staehelin, H. R. and Zeller, A.: Colorimetric and Enzymic Estimation of Histidine. Zeit. physiol. Chem. 1941, **270**, 158–164. from Chem. Abs. 1943, **37**, 401–402.

205. Eegriwe, E.: Reaktionen und Reagenzien zum Nachweis organischer Verbindungen. I. Zeit. f. Anal. Chem. 1932, **89**, 121–125.

206. Eegriwe, E.: Reaktionen und Reagenzien zum Nachweis organischer Vergindungen. II. Zeit. f. Anal. Chem. 1933, **95**, 323–327.

207. Ehrlich, F. and Wendel, A.: Zur Kenntniss der Leucinfraktion des Eiweisses. Biochem. Zeit. 1908, **8**, 399–437.

208. Elson, L. A. and Morgan, W. T. J.: A Colorimetric Method for the Determination of Glucosamine and Chondrosamine. Biochem. J. 1933, **27**, 1824–1828.

209. Elsworth, F. F. and Phillips, H.: The Action of Sulfites on the Cystine Disulfide Linkages in Wool. 2. The Influence of Temperature, Time, and Concentration on the Reaction. Biochem. J. 1941, **35**, 135–143.

210. Engeland, R.: Über Hydrolyse von Casein und den Nachweiss der dabei entstandenen Monoaminosäuren. Ber deut. chem. Gesel. 1909. **42**, 2962–2969.

211. Engeland, R.: Nachweiss und Bestimmung der Monoaminosäuren. Zeit. physiol. Chem. 1922, **120**, 130–140.

212. Engeland, R. and Bastian, A.: Technique de dosage de la proline dans les hydrolysats protéiques. Bull. Soc. Chim. biol. 1937, **19,** 1126–1128.
213. Erickson, B. N., Hoffman, O., Beach, E. F., Williams, H. H. and Macy, I. G.: Correlated Hematologic, Physical, and Chemical Studies of the Blood and Stroma in Polycythemia and Beef Embryo. J. Lab. Clin. Med. 1941, **26,** 1492–1499.
214. Evans, J. S. and Hauschildt, J. D.: The Influence of Enzymes upon the Activity of Gonadatropin of Pregnant Mare Serum. J. Bio¹ Chem. 1942, **145,** 335–339 and private communication.
215. Fabinyi, R.: Verfahren zur Darstellung von Farbstoffen aus Salicylaldehyd und Ketonverbindungen. Chem. Centralblatt 1900, (2), 301–302.
216. Fabinyi, R.: Verfahren zur Darstellung eines neuen Seidenfarbstoffes. Chem. Centralblatt 1900, (2), 302–303.
217. Fearon, W. R.: The Carbamido Diacetyl Reaction: A Test for Citrulline. Biochem. J. 1939, **33,** 902–907.
218. Fieser, L. F.: Experiments in Organic Chemistry, D. C. Heath and Co., New York, 1935, 369 pages.
219. Fischer, E.: Ueber die Ester der Aminosäuren. Ber. deut. chem. Gesel. 1901, **34,** 433–454.
220. Fischer, E.: Ueber die Hydrolyse des Caseins durch Salzsäure. Zeit. physiol. Chem. 1901, **33,** 151–176.
221. Fischer, E. and Bergell, P.: Ueber die β-Naphthalinsulfoderivate der Aminosäuren. Ber. deut. chem. Gesel. 1902, **35,** 3779–3787.
222. Fischer, E., Levene, P. A. and Aders, R. H.: Ueber die Hydrolyse des Leims. Zeit. physiol. Chem. 1902, **35,** 70–79.
223. Fischer, E.: Notizen. II. Quantitative Bestimmung des Glycocolls. Zeit. physiol. Chem. 1902, **35,** 227–230.
224. Fischer, E. and Dörpinghaus, T.: Hydrolyse des Horns. Zeit. physiol. 1902, **36,** 462–477.
225. Fleming, R.: A Sensitive Reaction for Cysteine. Biochem. J. 1930, **24,** 965–966.
226. Fleitmann, T.: Ueber die Existenz eines schwefelfreien Proteins. Leibig's Ann. 1847, **61,** 121–126.
227. Földes, F.: Das Vorkommen des Histidins im menschlichen Urin. Biochem. Zeit. 1935–36, **283,** 199–209.
228. Folin, O. and Macallum, A. B., Jr.: A New Method for the (Colorimetric) Determination of Uric Acid in Urine. J. Biol. Chem. 1912–13, **13,** 363–369.
229. Folin, O. and Wright, L. E.: A Simplified Macro-Kjeldahl Method for Urine. J. Biol. Chem. 1919, **38,** 461–464.
230. Folin, O.: A System of Blood Analysis. A New Colorimetric Method for the Determination of the Amino-Acid Nitrogen in Blood. J. Biol. Chem. 1922, **51,** 377–391.
231. Folin, O. and Looney, J. M.: Colorimetric Methods for the Separate

Determination of Tyrosine, Tryptophane, and Cystine in Proteins. J. Biol. Chem. 1922, **51**, 421–434.

232. Folin, O. and Ciocalteu, V.: On Tyrosine and Tryptophane Determinations in Proteins. J. Biol. Chem. 1927, **73**, 627–650.

233. Folin, O. and Marenzi, A. D.: Tyrosine and Tryptophane Determinations in One-Tenth Gram of Protein. J. Biol. Chem. 1929, **83**, 89–102.

234. Folin, O. and Marenzi, A. D.: An Improved Colorimetric Method for the Determination of Cystine in Proteins. J. Biol. Chem. 1929, **83**, 103–108.

235. Folin, O and Marenzi, A. D.: The Preparation of Uric Acid Reagent Completely Free from Phenol Reagent. J. Biol. Chem. 1929, **83**, 109–113.

236. Folin, O.: The Preparation of Sodium Tungstate Free from Molybdate, together with a Simplified Process for the Preparation of a Correct Uric Acid Reagent (and some Comments). J. Biol. Chem. 1934, **106**, 311–314.

237. Folley, S. J.: An Improved Design of the Van Slyke Apparatus for the Estimation of Amino-Nitrogen. Biochem. J. 1930, **24**, 961–964.

238. Fontaine, T. D., Olcott, H. S. and Lowy, A.: Amino Acid Composition of Cottonseed Globulin Preparations. Ind. and Eng. Chem. 1942, **34**, 116–118.

239. Foreman, F. W.: Quantitative Estimation of Aspartic and Glutaminic Acids in the Products of Protein Hydrolysis. Biochem. J. 1914, **8**, 463–480.

240. Foreman, F. W.: A New Method for Preparing Esters of Amino Acids. Composition of Caseinogen. Biochem. J. 1919, **13**, 378–397.

241. Fosse, R.: L'Uréa. Les Presses Universitaires de France, Paris 1928, 303 pages.

242. Foster, G. L. and Shemin, D.: *l*-Histidine Monohydrochloride. Organic Syntheses 1938, **18**, 43–46.

243. Freyberg, R. H., Block, W. D. and Fromer, M. F.: A Study of Sulfur Metabolism and the Effect of Sulfur Administration in Chronic Arthritis. J. Clin. Invest. 1940, **19**, 423–435.

244. Friedemann, T. E. and Kendall, A. I.: The Determination of Lactic Acid. J. Biol. Chem. 1929, **82**, 23–43.

245. Fromageot, C. and Heitz, P.: Méthode de dosage spécifique de l'alanine et de la somme sérine et acide aspartique. Microchemica acta. 1938, **3**, 52–67.

246. Fromageot, C. and Heitz, P.: Méthode de dosage spécifique de la valine et de la leucine dans un mélange d'acides aminés quelconques. Enzymologia 1939, **6**, 258–270.

247. Fromageot, C. and Mourgue, M.: Dosage de la valine et de la leucine dans les matières protéiques. Enzymologia 1941, **9**, 329–336.

248. Fürth, O.: Kritisches und Experimentelles über die Ermittlung des Tyrosingehaltes von Proteinen. II. Biochem. Zeit. 1924, **146**, 259–274.

249. Fürth, O. and Dische, Z.: Kritisches und Experimentelles über die Tryptophanbestimmung in Proteinen. Biochem. Zeit. 1924, **146,** 275–296.

250. Fürth, O. and Fischer, A.: Über die Ermittlung des Tyrosingehalts von Proteinen. III. Biochem. Zeit. 1924, **154,** 1–23.

251. Fürth, O. and Deutschberger, O.: Über den Arginingehalt einiger Proteine sowie normaler und amyloidhaltiger Organe. Biochem. Zeit. 1927, **186,** 139–154.

252. Fürth, O. and Minnibeck, H.: Methodische Versuche über den Prolin- und Oxyprolingehalt einiger Proteine. Biochem. Zeit. 1932, **250,** 18–34.

253. Fürth, O., Scholl, R. and Herrmann, H.: Über eine Mikrobestimmung des Alanins in Proteinen. Biochem. Zeit. 1932, **251,** 404–417.

254. Fujiwara, H. and Kataoka, E.: Kritische Untersuchungen über die Folin-Denisschen kolorimetrischen Verfahren. Zeit. physiol. Chem. 1933, **216,** 133–137.

255. Gaunt, W. E.: Amino Acids and the Human Being. Nature 1942, **149,** 666–667. ·

256. Gerngross, O., Voss, K. and Herfelt, T.: Über eine hochempfindliche, streng tyrosinspezifische Farbreaktion auf *para*-substituierter Phenole. Der Tyrosin-Gehalt verschiedener Proteine, insbesondere von Kollagen und Gelatine. Ber. deut. chem. Gesel. 1933, **66,** 435–442.

257. Gilson, L. E., Tytell, A. A., Logan, M. A. and Perkins, P.: Oxalic Acid Hydrolysate of Casein for Bacteriological Purposes. Federation Proceedings 1942, **1,** 112.

258. Giral, J. and Craviota, R. O.: Threonine in Maize. Ciencia 1941, **2,** 204–206, from Chem. Abs. 1942, **36,** 5483.

259. Goepfert, G. J.: A Micro-Determination of Succinic Acid. Biochem. J. 1940, **34,** 1012–1014.

260. Gordon, J. J. and Quastel, J. H.: Estimation of Lactic Acid in Biological Material by Oxidation with Ceric Sulfate. Biochem. J. 1939, **33,** 1332–1337.

261. Gordon, A. H., Martin, A. J. P. and Synge, R. L. M.: A study of the Partial Acid Hydrolysis of Some Proteins, with Special Reference to the Mode of Linkage of the Basic Amino-Acids. Biochem. J. 1941, **35,** 1369–1387.

261A. Gordon, S. A. and Wildman, S. G.: The Conversion of Tryptophane to a Plant Growth Substance by Conditions of Mild Alkalinity. J. Biol. Chem. 1943, **147,** 389–398.

261B. Gordon, A. H., Martin, A. J. P. and Synge, R. L. M.: Partition Chromatography in the Study of Protein Constituents. Biochem. J. 1943, **37,** 79–86.

261C. Gordon, A. H., Martin, A. J. P. and Synge, R. L. M.: The Amino Acid Composition of Gramicidin. Biochem. J. 1943, **37,** 86–92.

261D. Gordon, A. H., Martin, A. J. P. and Synge, R. L. M.: A Study of the Partial Hydrolysis of Cow-Hide Gelatin. Biochem. J. 1943, **37,** 92–102.

261E. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M.: The Amino Acid Composition of Tyrocidine, Biochem. J. 1943, 37, 313–318.

262. Gornall, A. G. and Hunter, A.: A Colorimetric Method for the Determination of Citrulline. Biochem. J. 1941, 35, 650–658.

263. Graff, S., Maculla, E. and Graff, A. M.: The Composition of the Tissue Proteins. II. The Estimation of Arginine. J. Biol. Chem. 1937, 121, 71–77.

264. Graff, S.: Maculla, E. and Graff, A. M.: The Composition of the Tissue Proteins. IV. The Estimation of Cystine. J. Biol. Chem. 1937, 121, 81–86.

265. Graff, S., Rittenberg, D. and Foster, G. L.: The Glutamic Acid of Malignant Tumors. J. Biol. Chem. 1940, 133, 745–752.

266. Greenstein, J. P. and Jenrette, W. V.: Chemical Studies on the Components of Normal and Neoplastic Tissues. II. The Nucleoprotein Fraction of Normal Animal Liver. J. Nat. Cancer Institute 1940, 1, 91–104.

267. Greenstein, J. P., Jenrette, W. V. and White, J.: Note on the Composition of the Nucleoprotein Fraction of Normal Liver and the Transplanted Hepatic Tumor in the Rat. J. Nat. Cancer Institute 1941, 2, 305–306.

268. Guest, G. H. and McFarlane, W. D.: A Study of the Colorimetric Determination of Pyrrole with Isatin and the Application of the Method to Biological Materials. Canad. J. Research 1939, 17B, 133–138.

269. Guest, G. H.: A Note on the Colorimetric Determination of Proline. Canad. J. Research 1939, 17B, 143–144.

270. Gulewitsch, W.: Ueber das Arginin. Zeit. physiol. Chem. 1899, 27, 178–215.

271. Gulland, J. M. and Morris, C. J. O. R.: Hydroxyglutamic Acid. J. Chem. Soc. (London). 1934, 1644–1649.

272. Gurevich, D. I.: The Tyrosine and Tryptophane Contents of Muscular Proteins of the White Leghorn Chick in Relation to Age and Sex. J. Physiol. U.S.S.R. 1939, 27, 615–617.

273. Gurin, S.: High Vacuum Distillation of N-Acyl Amino Acid and Polypeptide Esters. J. Amer. Chem. Soc. 1936, 58, 2104–2106.

274. Gurin, S. and Hood, D. B.: The Identification and Estimation of Hexoses in Polysaccharides and Glycoproteins by the Carbazole Methods. J. Biol. Chem. 1939, 131, 211–223.

275. Guthrie, J. D. and Allerton, J.: Use of Sulfur as a Reagent for the Estimation of the Sulfhydryl Groups of Egg Albumin. Contrib. Boyce Thompson Inst. 1941, 12, 103–109.

276. Hallett, L. T.: A Review of Organic Micro-Chemistry. Ind. and Eng. Chem., Anal. Ed. 1942, 14, 956–993.

277. Hamilton, T. S. and Nakamura, F. I.: The Cystine Content of Eleven Varieties of Soybeans. J. Agr. Research 1940, 61, 207–214.

278. Hanke, M. T. and Koessler, K. K.: Studies on Proteinogenous Amines. VII. The Quantitative Colorimetric Estimation of His-

tidine in Protein and Protein-Containing Matter. J. Biol. Chem. 1920, **43**, 527–542.

279. Hanke, M. T.: The Quantitative Estimation of Tyrosine and Histidine in Protein. A Method for Estimating Tyrosine in Protein-Containing Mixtures. J. Biol. Chem. 1925, **66**, 475–488.

280. Hanke, M. T.: The Histidine and and Tyrosine Content of a Number of Proteins. J. Biol. Chem. 1925, **66**, 489–493.

281. Hanke, M. T.: Determination of the Tyrosine Content of Proteins. J. Biol. Chem. 1928, **79**, 587–609.

282. Harden, A. and Norris, D.: The Diacetyl Reaction for Proteins. J. Physiol. 1911, **42**, 332–336.

283. Hart, E.: Ueber die quantitative Bestimmung der Spaltungs produkte von Eiweisskörpern. Zeit. physiol. Chem. 1901, **33**, 347–362.

284. Hausmann, W.: Ueber die Vertheilung des Stickstoffs in Eiweissmolekül. Zeit. physiol. Chem. 1899, **27**, 95–108.

285. Heffter, A.: Die reduzierenden Bestandteile der Zelle. Chem. Zentr. 1907, **3**, 822–824.

286. Heinrich, E.: The Protein Constituents and other Nitrogen Containing Substances in Certain Feeding Stuffs. The Amino Acid Content of Certain Fertilizers. Bodenkunde. u. Pflanzenernähr. 1941, **23**, 91–97, from Chem. Abs. 1942, **36**, 4926.

287. Hektoen, L. and Welker, W. H.: Immunological Differences of Crystalline Bence-Jones Proteins. Biochem. J. 1940, **34**, 487–489.

288. Hellerman, L., Chinard, F. P. and Ramsdell, P. A.: o-Iodosobenzoic Acid, a Reagent for the Estimation of Cysteine, Glutathione, and the Substituent Sulfhydryl Groups of Certain Proteins. J. Amer Chem. Soc. 1941, **63**, 2551–2553.

289. Herriott, R. M.: Reaction of Folin's Reagent with Proteins and Biuret Compounds in the Presence of Cupric Ion. Proc. Soc. Exp. Biol. and Med. 1941, **46**, 642–644.

290. Hess, W. C.: The Gasometric Method for the Estimation of Cysteine and Cystine. J. Biol. Chem. 1933, **100**, LIV.

291. Hess, W. C.: The Gasometric Determination of Cysteine and Cystine. J. Biol. Chem. 1933, **103**, 449–453.

292. Hess, W. C.: Variations in Amino Acid Content of Finger Nails of Normal and Arthritic Individuals. Proc. Amer. Soc. Biol. Chem., J. Biol. Chem. 1935, **109**, XLIII.

293. Hess, W. C. and Sullivan, M. X.: The Cystine Content of Deaminized Proteins. J. Biol. Chem. 1939, **128**, 93–99.

294. Hess, W. C., Sullivan, M. X. and Palmes, E. D.: The Colorimetric Determination of Cystine in Tobacco Mosaic Virus Protein. Proc. Soc. Exp. Biol. and Med. 1941, **48**, 353–355.

295. Hess, W. C. and Sullivan, M. X.: Canine Cystinuria. The Cystine Output on an Arachin Diet. J. Biol. Chem. 1942, **146**, 381–384.

296. Hewitt, L. F.: Separation of Serum Albumin into Two Fractions. Biochem, J. 1936, **30**, 2229–2236.

297. Hewitt, L. F.: Separation of Serum Albumin into Two Fractions.

II. Observations on the Nature of the Glycoprotein Fraction. Biochem. J. 1937, **31**, 360–366.

298. Hijikata, Y.: On the Cleavage Products of the Crystalline Lens. J. Biol. Chem. 1922, **51**, 155–164.

299. Hirohata, R.: Studies ǒn Protamine. II. Mugiline β. J. Biochem. (Japan) 1937, **25**, 519–524.

300. Hlaziwetz, H. and Habermann J.: Ueber die Proteïnstoffe. Liebig's Ann. 1873, **169**, 150–166.

301. Hoffmann, R.: Reaction auf Leucin und Tyrosin. Liebig's Ann 1853, **87**, 123–125.

302. Holiday, E. R.: Spectrophotometry of Proteins. I. Absorption Spectra of Tyrosine, Tryptophane, and their Mixture. II. Estimation of Tyrosine and Tryptophane in Proteins. Biochem. J. 1936. **30**, 1795–1803.

303. Holm, G. E. and Greenbank, G. R.: The Quantitative Determination of Tryptophane. J. Amer. Chem. Soc. 1923, **45**, 1788–1792.

304. Homer, A.: A Spectroscopic Examination of the Color Reactions of Certain Indol Derivatives and of the Urine of Dogs after their Administration. J. Biol. Chem. 1915, **22**, 345–368.

305. Homer, A.: A Method for the Estimation of the Tryptophane Content of Proteins, Involving the Use of Baryta as a Hydrolyzing Agent. J. Biol. Chem. 1915, **22**, 369–389.

306. Hopkins, F. G. and Cole, S. W.: On the Proteid Reaction of Adamkiewicz, with Contributions to the Chemistry of Glyoxylic Acid. Proc. Royal Soc. London, 1901, **68**, 21–33.

307. Hopkins, F. G. and Cole, S. W.: A Contribution to the Chemistry of Proteids. I. A Preliminary Study of a hitherto Undescribed Product of Tryptic Digestion. J. Physiol. 1901–2, **27**, 418–428.

308. Hopkins, F. G. and Savory, H.: A Study of Bence-Jones Protein and of the Metabolism in Three Cases of Bence-Jones Proteinuria. J. Physiol. 1911, **42**, 189–250.

309. Hopkins, F. G.: On Glutathione: A Reinvestigation. J. Biol. Chem. 1929, **84**, 269–320.

310. Hotchkiss, R. D.: The Chemical Nature of Gramicidin and Tyrocidine. J. Biol. Chem. 1941, **141**, 171–185.

311. Hunter, G.: Note on Knoop's Test for Histidine. Biochem. J. 1922, **16**, 637–639.

312. Hunter, A. and Borsook, H.: Nitrogen Distribution in Globin. J. Biol. Chem. 1923, **57**, 507–514.

313. Hunter, A. and Dauphinee, J. A.: The Arginase Method for the Determination of Arginine and its Use in the Analysis of Proteins. J. Biol. Chem. 1929–30, **85**, 627–665.

314. Hunter, A. and Pettigrew, J. B.: A Manometric Method for the Enzymatic Determination of Arginine. Enzymologia 1937, **1**, 341–352.

315. Ing. H. R. and Bergmann, M.: Semimicroestimation of Amino Acids. J. Biol. Chem. 1939, **129**, 603–607.

316. Jansen, B. C. P.: La Transformation d'Arginine en Créatine dans l'Organisme Animal. Arch. néerl. Physiol. 1916, 1, 618–624. ·

317. Jean, G.: Sur les microdosages colorimetriques de l'arginine en solution pure et dans les hydrolysates protéiques. Bull. Soc. Chim. biol. 1934, 16, 307–309.

318. Jensen, H., Wintersteiner, O. and du Vigneaud, V.: Studies on Crystalline Insulin. V. The Distribution of Nitrogen in Crystalline Insulin. J. Pharmacol. and Exp. Therap. 1928, 32, 397–411.

319. Jensen, H. and Wintersteiner, O.: Studies on Crystalline Insulin. XIV. The Isolation of Glutamic Acid. J. Biol. Chem. 1932, 97, 93–98.

320. Jensen, H. and Wintersteiner, O.: Studies on Crystalline Insulin. XVII. The Hydrolysis Products of Insulin. J. Biol. Chem. 1932, 98, 281–287.

321. Jervis, G. A., Block, R. J., Bolling, D. and Kanze, E.: Chemical and Metabolic Studies on Phenylalanine. II. The Phenylalanine Content of the Blood and Spinal Fluid in Phenylpyruvic Oligophrenia. J. Biol. Chem. 1940, 134, 105–113.

322. Johns, C. O. and Jones, D. B.: The Proteins of the Peanut, *Arachis Hypogaea*. III. The Hydrolysis of Arachin. J. Biol. Chem. 1918, 36, 491–500.

333. Johns, C. O., Finks, A. J. and Gersdorff, C. E. F.: Globulin of the Cocoanut, *Cocos Nucifera*. I. Preparation of Cocoanut Globulin. Distribution of the Basic Nitrogen in Cocoanut Globulin. J. Biol. Chem. 1919, 37, 149–153.

334. Johns, C. O. and Jones, D. B.: Some Amino-Acids from the Globulin of the Cocoanut as Determined by the Butyl Alcohol Extraction Method of Dakin. J. Biol. Chem. 1920, 44, 283–290.

335. Johns, C. O. and Waterman, H. C.: Some Proteins from the Mung Bean, *Phaseolus Aureus* Roxburgh. J. Biol. Chem. 1920, 44, 303–317.

336. Johnson, J. M.: A Comparison of the Optical Forms of Glutamic Acid from Normal and Cancerous Tissue. J. Biol. Chem. 1940, 132, 781–782.

337. Jolles, A.: Über eine neue Indikan-Reaktion. Zeit. physiol. Chem. 1913, 87, 310–312.

338. Jones, D. B. and Johns, C. O.: The Hydrolysis of Kafirin. J. Biol. Chem. 1918, 36, 323–334.

339. Jones, D. B. and Johns, C. O.: Hydrolysis of the Globulin of the Cocoanut, *Cocos Nucifera*. J. Biol. Chem. 1920, 44, 291–301.

340. Jones, D. B. and Johns, C. O.: Determination of the Monoamino-Acids in the Hydrolytic Cleavage Products of Lactalbumin. J. Biol. Chem. 1921, 48, 347–360.

341. Jones, D. B., Finks, A. J. and Gersdorff, C. E. F.: A Chemical Study of the Proteins of the Adsuki Bean, *Phaseolus Angularis*. J. Biol. Chem. 1922, 51, 103–114.

342. Jones, D. B., Gersdorff, C. E. F. and Moeller, O.: The Tryptophane

and Cystine Content of Various Proteins. J. Biol. Chem. 1924–25, **62**, 183–195.

343. Jones, D. B. and Moeller, O.: Some Recent Determinations of Aspartic and Glutamic Acids in Various Proteins. J. Biol. Chem. 1928, **79**, 429–441.

344. Jorpes, E.: The Protein Component of the Erythrocyte Membrane or Stroma. Biochem. J. 1932, **26**, 1488–1503.

345. Jorpes, E. and Thorén, S.: The Use of the Sakaguchi Reaction for the Quantitative Determination of Arginine. Biochem. J. 1932, **26**, 1504–1506.

346. Jorpes, E.: The Colorimetric Determination of Histidine. Biochem. J. 1932, **26**, 1507–1511.

347. Jukes, T. H. and Kay, H. D.: The Basic Amino Acids of Livetin J. Biol. Chem. 1932, **98**, 783–788.

348. Jukes, T. H.: The Fractionation of the Amino Acids of Livetin. J. Biol. Chem. 1933, **103**, 425–437.

349. Kandatu, M.: Muscle Protein, II. Amino Acids in Rabbit Muscle Protein. J. Agr. Chem. Soc. (Japan) 1941, **17**, 731–744, from Chem. Abs. 1942, **36**, 5521.

350. Kapeller-Adler, R.: Über eine neue Reaktion zur qualitativen und quantitativen Bestimmung des Phenylalanins. Biochem. Zeit. 1932, **252**, 185–200.

351. Kapeller-Adler, R.: Über eine neue Methode zur quantitativen Histidinbestimmung und über deren Anwendbarkeit zur Untersuchung von biologischen Flüssigkeiten, insbesondere von Gravidenharnen. Biochem. Zeit. 1933, **264**, 131–141.

352. Kapeller-Adler, R.: Über eine stufenphotometrische Bestimmung des Histidins. Biochem. Zeit. 1934, **271**, 206–208.

353. Kapfhammer, J. and Eck, R.: l-Oxyprolin und l-Prolin. Ihre Darstellung aus Eiweiss mit Hilfe der Reineckesäure. Zeit. physiol. Chem. 1927, **170**, 294–312.

354. Kaplansky, S. J.: Vergleichend-biochemische Untersuchung der Eiweisskörper des Gehirns. Aminosäurengehalt der Eiweisskörper des Gehirns bei verschiedenen Wirbeltieren. Arb. Timirjaseff Inst. Biol. Moscow 1935, 243–255.

355. Karrer, P., Smirnoff, A. P., Ehrensperger, H., van Slooten, J. and Keller, M.: Über Toxine. I. Zur Kenntnis des Ricins. Zeit. physiol. Chem. 1924, **135**, 128–166.

356. Kassell, B. and Brand, E.: The Photometric Determination of Cystine, Cysteine, Ascorbic Acid, and Related Compounds with Phosphotungstic Acid. J. Biol. Chem. 1938, **125**, 115–129.

357. Kassell, B. and Brand, E.: The Determination of Methionine, Cysteine, and Sulfate in Proteins after Hydrolysis with Hydriodic Acid. J. Biol. Chem. 1938, **125**, 145–159.

358. Kendall, A. I. and Friedemann, T. E.: The Quantitative Determination of Alanine. J. Infect. Diseases 1930, **47**, 171–175.

359. Kendrick, A. B. and Hanke, M. E.: The Use of Iodine and other

Modifications in the Van Slyke Manometric Amino Nitrogen Method. J. Biol. Chem. 1937, **117**, 161–174.

360. Kiech, V. C., Luck, J. M. and Smith, A. E.: Studies on Arginine I. The Rate of Catabolism of Arginine in Rats, Including a Method for the Determination of Arginine in' Biological Material. J. Biol. Chem. 1931, **90**, 677–696.

361. Kiesel, A.: Zur Methodik der Fällung von Basen als Silberverbindungen. Zeit. physiol. Chem. 1926, **161**, 147–148.

362. Kiesel, A. and Znamenskaja, M.: Studien im Gebiete der Strukturchemie der Eiweisskörper I. Über Ringschliessung und Sauerstoffanreicherung beim Methoxylieren des Glycinins. Zeit. physiol. Chem. 1932, **213**, 89–109.

363. Kiesel, A. and Kusmin, S.: Über chemische Vorgänge bei der Hitzdenaturierung von Eiweiss. Zeit. physiol. Chem. 1936, **238**, 145–148.

364. Kik, M. C.: Nutritive Studies of Rice and its By-Products. Arkansas Agri. Exp. Station 1942, *Bull. 416*, 1–28.

365. Kingston, H. L. and Schryver, S. B.: Investigations on Gelatin. III. The Separation of the Products of Hydrolysis of Gelatin by the Carbamate Method. Biochem. J. 1924, **18**, 1070–1078.

366. Klabunde, H. K.: Note on the Preparation of Hydroxyproline. J. Biol. Chem. 1931, **90**, 293–295.

367. Klein, D.: An Improved Apparatus for the Determination of Amino Groups. J. Biol. Chem. 1911–12, **10**, 287–289.

368. Klein, G. and Linser, H.: Colorimetrische Methode zur quantitativen Bestimmung von Glykokoll. Zeit. physiol. Chem. 1932, **205**, 251–258.

369. Kleinschmitt, A.: Hydrolyse des Hordeins. Zeit. physiol. Chem. 1907–08, **54**, 110–118.

370. Knight, C. A. and Stanley, W. M.: Aromatic Amino Acids in Strains of Tobacco Mosaic Virus and in the Related Cucumber Viruses 3 and 4. J. Biol. Chem. 1941, **141**, 39–49.

371. Knight, C. A.; Basic Amino Acids in Strains of Tobacco Mosaic Virus. J. Amer. Chem. Soc. 1942, **64**, 2734–2736.

372. Knoop, F.: Eine Farbenreaktion des Histidins. Beitr. Chem. Physiol. und Pathol. 1908, **11**, 356.

373. Koch, F.: On the Presence of Histidine in Pig Thyreoglobulin. J. Biol. Chem. 1911, **9**, 121–122.

374. Koehler, A. E.: Modification of the Van Slyke Method for Determining Arginine. J. Biol. Chem. 1920, **42**, 267–268.

375. Koessler, K. K. and Hanke, M. T.: Studies on Proteinogenous Amines. II. A Microchemical Colorimetric Method for Estimating Imidazole Derivatives. J. Biol. Chem. 1919, **39**, 497–519.

376. Kollmann, G.: Versuche zur Bestimmung des Phenylalanins auf oxydativen Wege. Biochem. Zeit. 1928, **194**, 1–14.

377. Komm, E.: Beitrag zur Tryptophan-Aldehydreaktion. V. Methode zur quantitativen Tryptophanbestimmung und Ermittlung des Tryptophangehaltes einiger Proteine. Zeit. physiol. Chem. 1926, **156**, 202–217.

378. Kossel, A.: Ueber die Constitution der einfachsten Eiweisstoffe. Zeit. physiol. Chem. 1898, **25**, 165–189.

379. Kossel, A. and Kutscher, F.: Beiträge zur Kenntniss der Eiweisskörper. Zeit. physiol. Chem. 1900–01, **31**, 165–214.

380. Kossel, A. and Patten, A. J.: Zur Analyze der Hexonbasen. Zeit. physiol. Chem. 1903, **38**, 39–45.

381. Kossel, A. and Pringle, H.: Über Protamine und Histone. Zeit. physiol. Chem. 1906, **49**, 301–321.

383. Kossel, A. and Weiss, F.: Über die Einwirkung von Alkalien auf Proteinstoffe. Zeit. physiol. Chem. 1910, **68**, 165–169.

384. Kossel, A. and Gross, R. E.: Über die Darstellung und quantitative Bestimmung des Arginins. Zeit. physiol. Chem. 1924. **135**, 167-174.

385. Kossel, A. and Staudt, W.: Über die quantitative Bestimmung von Arginin und Histidin. Zeit. physiol. Chem. 1926, **156**, 270–274.

386. Kossel, A. and Staudt, W.: Die Gewinnung Eines Argininpeptid aus Clupein. Zeit. physiol. Chem. 1927, **170**, 91–105.

387. Kovács, E.: Vergleichende physikalische und chemische Untersuchungen an Cascinen verschiedener Tierarten. Biochem. Zeit. 1940, **306**, 74–76.

388. Kraus, I.: Colorimetric Determination of Tryptophane by the Vanillin-HCl Reaction and the Quantitative Separation from Indole and Skatole. J. Biol. Chem. 1925, **63**, 157–178.

389. Kraus-Ragins, I.: The Further Application of the Vanillin-Hydrochloric Acid Reaction in the Determination of Tryptophane in Proteins. J. Biol. Chem. 1928, **80**, 543–556.

390. Krebs, H. A.: The Role of Fumarate in the Respiration of *Bacterium Coli Commune*. Biochem. J. 1937, **31**, 2095–2124.

391. Krijgsman, B. J. and Bouman, E. F.: Beiträge zur Methodik der Cystinbestimmung. Arch. Neérl. Physiol. 1933, **18**, 458–471.

392. Krishnaswamy, T. K.: Estimation of Cystine by Nitroprusside. Proc. Indian Acad. Sci. 1942, **15A**, 135–138, from Chem. Abs. 1942, **36**, 4058.

393. Kuhn, R. and Desnuelle, P.: Über die Aminosäuren des gelben Ferments. Ber. deut. chem. Gesel. 1937, **70**, 1907–1926.

394. Kuhn, R. and Desnuelle, P.: Über die Isolierung von Arginin, Histidin, Lysin, Glutaminsäure und Asparaginsäure aus gelbem Ferment. Zeit. physiol. Chem. 1938, **251**, 19–22.

395. Kuhn, R., Birkofer, L. and Quackenbush, F. W.: Iodometrische Titration von SH-Gruppen; Mikromethode zur Bestimmung von Cystein und Methionin in Proteinen. Ber. deut. chem. Gesel. 1939, **72**, 407–416.

396. Kuhn, R., Sørensen, N. A. and Birkofer, L.: Über die Eisenproteide der Milz; der Bauplan des Ferritins. Ber. deut. chem. Gesel. 1940, **73B**, 823–837, from Chem. Abs. 1941, **35**, 3263.

397. Laine, T.: Micro-determination of Leucine by Ninhydrine Method. Suomen Kemistilehti 1938, **11B**, 28.

398. Laine, T.: Applications of certain Micro-Methods in the Analysis of Zein. Suomen Kemistilehti 1939, **12B**, 23.

399. Lang, K.: Über den Mechanismus der Diacetylreaktion von Guanidinen; ihre Umformung und Anwendung zur kolorimetrischen Bestimmung von Kreatin und Argirin. Zeit. physiol. Chem. 1932, **208**, 273–280.

400. Lang, K.: Eine Mikromethode zur Bestimmung des Prolins und des Oxyprolins. Zeit. physiol. Chem. 1933, **219**, 148–154.

401. Lang, K.: Eine Mikromethode zur Bestimmung des Histidins insbesondere ein Verfahren zur Trennung von Histidin und Tyrosin. Zeit. physiol. Chem. 1933, **222**, 3–5.

402. Langley, W. D.: Urinary Histidine. Determination of Histidine in Urine. Histidine in Normal and in Pregnancy Urine. J. Biol. Chem. 1941, **137**, 255–265.

403. Lapraik, W.: Ueber die Absorptionsspectra einiger Chromverbindungen. J. prakt. Chem. 1893, **47**, 305–342.

404. Larmour, R. K.: A Comparative Study of the Glutelins of the Cereal Grains. J. Agricul. Research 1927, **35**, 1091–1120.

405. Lautenschläger, C. L.: Über die titrimetrische Bestimmung des Histidins und anderer Imidazolderivate. Zeit. physiol. Chem. 1918, **102**, 226–243.

406. Lavine, T. F.: The Iodometric Determination of Cysteine. J. Biol. Chem. 1935, **109**, 141–145.

407. Lavine, T. F.: A New Method for the Estimation of Methionine. Federation Proceed. 1942, **1**, 120–121.

408. Lavine, T. F.: An Iodometric Determination of Methionine. J. Biol. Chem. 1943, **151**, 281–297.

409. Lawrow, D.: Ueber die Spaltungsprodukte des Histons von Leucocyten. Zeit. physiol. Chem. 1899, **28**, 388–398.

410. Leavenworth, C. S.: Note on the Basic Amino Acids Yielded by Casein. J. Biol. Chem. 1924, **61**, 315–316.

411. Lee W. C. and Lewis, H. B.: The Effect of Fasting, Refeeding, and of Variations in the Cystine Content of the Diet on the Composition of the Tissue Proteins of the White Rat. J. Biol. Chem. 1934, **107**, 649–659.

412. Lefevre, C, and Desgrez, P.: Oxidation of Sulfur in Organic Chemistry, II. Determination of Methionine. J. pharm. chim. 1941, **1**, 341–349, from Chem. Abs. 1941, **35**, 7997.

413. Leipert, T. and Alcock, R.: Retikuloendotheliales System und Aminosäurenstoffwechsel. Biochem. Zeit. 1934, **270**, 99–103.

414. Levene, P. A.: Glycocoll Picrate. J. Biol. Chem. 1905–06, **1**, 413–414.

415. Levene, P. A. and Van Slyke, D. D.: The Leucin Fraction of Proteins. J. Biol. Chem. 1909, **6**, 391–418.

416. Levene, P. A. and Van Slyke, D. D.: The Leucin Fraction in Casein and Edestin. J. Biol. Chem. 1909, **6**, 419–430.

417. Levene, P. A. and Van Slyke, D. D.: The Separation of d-Alanine and d-Valine. J. Biol. Chem. 1913–14, **16**, 103–120.

418. Lewis, H. B. and Frayser, L.: The Metabolism of Sulfur. XXII. The Cystine Content of the Hair and Nails of Cystinurics. J. Biol. Chem. 1935, **110**, 23–27.

419. Li, C. H., Lyons, W. R. and Evans, H. M.: Studies on Pituitary Lactogenic Hormone. VI. Molecular Weight of the Pure Hormone. J. Biol. Chem. 1941, **140**, 43–53.

420. Lieben, F. and Loo, Y. C.: On the Liberation of Free Amino Nitrogen from Proteins in the Van Slyke Apparatus. J. Biol. Chem. 1942, **145**, 223–228.

421. Lizzitzin, M. A. and Alexandrowskaja, N. S.: Über die chemische Zusammensetzung der Störprotamine. Zeit. physiol. Chem. 1936, **238**, 54–58.

422. Lock, K. and Thomas, K.: Untersuchungen über den Gehalt der Blutplasmaproteine an basischen Bestandteilen. Zeit. physiol. Chem. 1913, **87**, 74–81.

423. Lucas, C. C. and King, E. J.: The Iodimetric Titration of Cysteine and Allied Substances. Biochem. J. 1932, **26**, 2076–2089.

424. Lucas, C. C. and Beveridge, J. M. R.: The Analysis of Hair Keratin. 1. A Method for the Quantitative Removal of Cystine from Keratin Hydrolysates. Biochem. J. 1940, **34**, 1356–1366.

425. Lugg, J. W. H.: The Application of Phospho-18-Tungstic Acid (Folin's Reagent) to the Colorimetric Determination of Cysteine, Cystine and Related Substances. I. The Reduction of Phospho-18-Tungstic Acid by Various Substances. Biochem. J. 1932, **26**, 2144–2159.

426. Lugg, J. W. H.: The Application of Phospho-18-Tungstic Acid (Folin's Reagent) to the Colorimetric Determination of Cysteine, Cystine and Related Substances. II. The Determination of Sulfhydryl Compounds and Disulfides already Existing in Solution. Biochem. J. 1932, **26**, 2160–2165.

427. Lugg, J. W. H.: Sullivan's Reaction for the Quantitative Determination of Cysteine and Cystine. Biochem. J. 1933, **27**, 668–673.

428. Lugg, J. W. H.: Some Sources of Error in the Estimation of Cysteine and Cystine in Complex Materials when Acid Hydrolysis is Employed. Biochem. J. 1933, **27**, 1022–1029.

429. Lugg, J. W. H.: On the Use of Mercuric Salts and Nitrous Acid in the Colorimetric Determination of Tyrosine and Tryptophane Present in Solution. Biochem. J. 1937, **31**, 1423–1433.

430. Lugg, J. W. H.: Investigations of Sources of Error in the Estimation of Tyrosine and Tryptophane in Complex Materials which are Associated with Hydrolysis. Biochem. J. 1938, **32**, 775–783.

431. Lugg, J. W. H.: Preparation of some Protein Samples from the Fresh Leaves of Plants and the Sulfur Distribution of the Preparations. Biochem. J. 1938, **32**, 2114–2122.

432. Lugg, J. W. H.: The Amide, Tyrosine and Tryptophane Contents and the Sulfur Distributions (Cystine plus Cysteine and Methionine Contents), of some Plant Leaf Protein Preparations. Biochem. J. 1938, **32**, 2123–2128.

433. Lugg, J. W. H.: The Representativeness of Extracted Samples and the Efficiency of Extraction of Protein from the Fresh Leaves of Plants; and some Partial Analyses of the Whole Proteins. Biochem. J. 1939, 33, 110–122.,

433A. Lugg, J. W. H.: Further Studies of Cryptogam Tissue Protein Composition and Tissue Function in Plants. Biochem. J. 1943, 37, 132–137.

433B. Lyman, C. M., Kuiken, K. A., Norman, W. H. and Hale, F.: Microbiological Determination of Amino Acids. Science 1943, 48, 266 and J. Biol. Chem. 151, 615–26 (1943).

434. Macpherson, H. T.: Modified Procedures for the Colorimetric Estimation of Arginine and Histidine. Biochem. J. 1942, 36, 59–63.

435. Malaprade, M. L.: Oxydation de quelques polyalcools per l'acide periodique. Applications. Comp. rend. Acad. Sciences 1928, 186, 382–384.

436. Marenzi, A. D.: Microméthode Permettant de Déterminer la Cystine des Protéines. Comp. rend. Soc. Biol. 1930, 104, 405–407.

437. Martin, A. J. P. and Synge, R. L. M.: Separation of the Higher Monoaminoacids by Counter-Current Liquid-Liquid Extraction: The Amino Acid Composition of Wool. Biochem. J. 1941, 35, 91–121.

438. Martin, A. J. P. and Synge, R. L. M.: Some Applications of Periodic Acid to the Study of the Hydroxyamino-Acids of Protein Hydrolysates. Biochem. J. 1941, 35, 294–314.

439. Martin, A. J. P. and Synge, R. L. M.: A New Form of Chromatogram Employing Two Liquid Phases. I. A Theory of Chromatography. II. Application to the Micro-Determination of the Higher Mono-Amino Acids in Proteins. Biochem. J. 1941, 35, 1358–1368.

440. May, C. E. and Rose, E. R.: The Tryptophane Content of some Proteins. J. Biol. Chem. 1922, 54, 213–216.

441. Mazur, A.: The Sulfur Distribution and Basic Amino Acids of Limulus Hemocyanin. J. Biol. Chem. 1937, 118, 631–634.

442. Mazur, A. and Clarke, H. T.: The Amino Acids of Certain Marine Algae. J. Biol. Chem. 1938, 123, 729–740.

443. Mazur, A. and Clarke, H. T.: Chemical Components of Some Autotropic Organisms. J. Biol. Chem. 1942, 143, 39–42.

444. McCarthy, T. E. and Sullivan, M. X.: A New and Highly Specific Colorimetric Test for Methionine. J. Biol. Chem. 1941, 141, 871–876.

445. McChesney, E. W.: Determination of Lactic Acid in Presence of Certain Interfering Substances. Proc. Soc. Exp. Biol. and Med. 1934, 32, 94–95.

446. McChesney, E. W.: The Determination of Alanine in Biological Materials. J. Elisha Mitchell Sci. Soc. 1935, 51, 147–150.

447. McFarlane, W. D. and Fulmer, H. L.: The Colorimetric Determination of the Tyrosine and Tryptophane Content of Various Crude Protein Concentrates. Biochem. J. 1930, 24, 1601–1610.

448. McFarlane, W. D., Fulmer, H. L. and Jukes, T. H.: Studies in Embryonic Mortality in the Chick. I. The Effect of Diet upon the Nitrogen, Amino-Nitrogen, Tyrosine, Tryptophane, Cystine, and Iron Content of the Proteins and on the Total Copper of the Hen's Egg. Biochem. J. 1930, **24**, 1611–1631.

449. McFarlane, W. D. and Guest, G. H.: A New Colorimetric Method for the Determination of Hydroxyproline and its Application to Gelatin Hydrolysates. Canad. J. Research, Sec. B. 1939, **17**, 139–142.

450. Miller, B. F. and Muntz, J. A.: A Method for the Estimation of Ultramicroquantities of Lactic Acid. J. Biol. Chem. 1939, **126**, 413–421.

451. Miller, E. J.: The Basic Amino-Acids of Typical Forage Grass Proteins. Biochem. J. 1935, **29**, 2344–2350.

452. Miller, E. J.: The Amino-Acid of Cocksfoot Protein with Special Reference to the Dicarboxylic Acids and Proline. Biochem. J. 1936, **30**, 273–279.

453. Miller, F. M. with Lyons, R. E.: Color Reactions of Amino Acids with Phenols and Hypochlorites, Including New Tests for Tryptophane and Phenylalanine. Proc. Indiana Acad. Sciences 1934, **43**, 132–135.

454. Miller, F. M. with Lyons, R. E.: Contributions to the Chemistry of Tryptophane. Proc. Indiana Acad. Sciences 1936, **45**, 151–156.

455. Miller, G. L. and du Vigneaud, V.: The Cystine Content of Insulin. J. Biol. Chem. 1937, **118**, 101–110.

456. Millon, M. E.: Sur un réactif propre aux composés protéiques. Comp. rend. Acad. Sciences 1849, **28**, 40–42.

457. Milone, H. S. and Sullivan, M. X.: A Study of the Cystine and Tryptophane Content of Normal and Pathological Sera. Med. Annals, Dist. of Columbia 1938, **7**, 335–340.

458. Milone, H. S. and Everitt, E. L.: Estimation of Tryptophane Content of Various Proteins. Proc. Soc. Exp. Biol. and Med. 1942, **51**, 82–83.

459. Mirsky, A. E. and Anson, M. L.: Protein Sulfhydryl Groups. Proc. Soc. Exp. Biol. and Med. 1930–31, **28**, 170–171.

460. Mirsky, A. E. and Anson, M. L.: Sulfhydryl and Disulfide Groups of Proteins. I. Methods of Estimation. J. Gen. Physiol. 1934–35, **18**, 307–323.

461. Mirsky, A. E. Private Communication, September, 1942.

462. Mitchell, H. H. and Hamilton, T. S.: The Biochemistry of the Amino Acids.; Chemical Catalogue Co., New York, 1929, 619 pages.

463. Mörner, K. A. H.: Zur Kenntniss der Bindung des Schwefel in den Proteinstoffen. Zeit. physiol. Chem. 1901–02, **34**, 207–338.

464. Mohler, M. E.: Recherche de l'acide benzoique dans les substances alimentaires. Bull. soc. Chim. 1890, **3**, 414–416.

465. Moore, S., Stein, W. H. and Bergmann, M.: Protein Constituent Analysis by the Solubility Method. Chem. Rev. 1942, **30**, 423–432.

465A. Moore, S. and Stein, W. H.: Determination of Amino Acids by the Solubility Product Method. J. Biol. Chem. 1943, **150,** 113–130.

466. Mourot, G. and Hoffer, O.: Dosage de l'arginine et de l'histidine. Bull. soc. chim. Biol. 1938, **2ρ,** 274–292.

467. Mulder, G. J.: Zusammensetzung von Fibrin, Albumin, Leimzucker, Leucin, usew. Liebig's Ann. 1838, **28,** 73–82.

468. Müller, E.: Die Nitranilsäure als Basenfällungsmittel. Zeit. physiol. Chem. 1941, **268,** 245–250.

469. Murrill, W. A., Block, W. D. and Newburgh, L. H.: Analysis of Urinary Proteins and Various Fractions of Human and Pig Serum Protein. J. Biol. Chem. 1940, **133,** 521–527.

470. Murrill, W. A. and Block, W. D.: Constancy of Chemical Composition of Serum Proteins Regenerated on Various Dietary Regimes. Arch. of Biochem. 1943, **1,** 365–368.

471. Narayana, N. and Sreenivasaya, M.: Characterisation of very small Quantities of Proteins by Van Slyke's Method. Biochem. J. 1928, **22,** 1135–1137.

472. Nasse, O.: Ueber die Verwendbarkeit des Millon'schen Reagens. Arch. f. ges. Physiol. 1901, **83,** 361–368.

473. Neuberg, C. and Kerb, J.: Ueber ein Fällungsmittel für Aminosäuren. Biochem. Zeit. 1912, **40,** 498–512.

474. Neuberger, A. and Sanger, F.: The Nitrogen of the Potato. Biochem. J. 1942, **36,** 662–671.

475. Nicolet, B. H. and Saidel, L. J.: The Hydroxyamino Acids of Silk Proteins. J. Biol. Chem. 1941, **139,** 477–478.

476. Nicolet, B. H. and Shinn, L. A.: The Determination of Serine by the Use of Periodate. J. Biol. Chem. 1941, **139,** 687–692.

476A. Nicolet, B. H. and Shinn, L. A.: A Determination of the Hydroxy Amino Acids of Insulin. J. Amer. Chem. Soc. 1941, **63,** 1486.

477. Nicolet, B. H. and Shinn, L. A.: Does Hydroxyglutamic Acid Occur in Milk Proteins. J. Biol. Chem. 1942, **142,** 139–146.

478. Nicolet, B. H.: Determination of Serine, Threonine, and Sugars (Including Methyl Pentoses) in Mixtures. Private Communication, April, 1942.

479. Nicolet, B. H., Shinn, L. A. and Saidel, L. J.: The Reaction of Phosphoproteins with Alkali and Acid. Amer. Chem. Soc. Abstracts 1942. September, 22–23B.

480. Nicols, R. R. and Eckert, H. W.: A New Micro Colorimetric Method for the Determination of Tryptophane. New York State Dept. of Health, Ann. Rept. 1941, 26–27 and Eckert, W. H.; J. Biol. Chem. 1943, **148,** 205–212.

481. Oesper, R. E. and Deasy, C. L.: The Preparation of Lead Tetraacetate. J. Amer. Chem. Soc. 1939, **61,** 972–973.

482. Okuda, Y.: A New Method for the Determination of Cystine in Proteins (The Iodine Method). J. Biochem. (Japan) 1925, **5,** 217–227.

483. Onslow, H.: On the Stability of Tryptophan in Baryta Hydrolysis. Biochem. J. 1921, **15,** 383–391.

484. Onslow, H.: On the Nature of the Substances Precipitated by Mercuric Sulfate from Hydrolyzed Caseinogen, with Reference to the Estimation and Isolation of Tryptophane. Biochem. J. 1921, **15**, 392–399.

485. Onslow, H.: A Method of Estimating the Tryptophane Content of Caseinogen Based on Determinations of the Nitrogen Values of the Mercuric Sulfate Precipitate. Biochem. J. 1924, **18**, 63–84.

486. Opsahl, J. C. and Arnow, L. E.: The Optical Configuration of Glutamic Acid Isolated from Casein Hydrolysates by Six Procedures. J. Amer. Chem. Soc. 1942, **64**, 2035–2039.

487. Orglmeister, G.: Über die Bestimmung des Arginins mit Permanganat. Beitr. chem. Physiol. und Path. 1905, **7**, 21–30.

488. Osborne, T. B. and Gilbert, R. D.: The Proportion of Glutaminic Acid Yielded by Various Vegetable Proteins when Decomposed by Boiling with Hydrochloric Acid. Amer. J. Physiol. 1906, **15**, 333–356.

489. Osborne, T. B. and Clapp, S. H.: The Chemistry of the Protein Bodies of the Wheat Kernel. III. Hydrolysis of the Wheat Proteins. Amer. J. Physiol. 1906–07, **17**, 231–265.

490. Osborne, T. B. and Clapp, S. H.: Hydrolysis of the Proteins of Maize, *Zea Mays.* Amer. J. Physiol. 1907–08, **20**, 477–493.

491. Osborne, T. B. and Heyl, F. W.: Hydrolysis of Vicilin from the Pea *(Pisum Sativum).* J. Biol. Chem. 1908–09, **5**, 187–195.

492. Osborne, T. B. and Heyl, F. W.: Hydrolysis of Legumelin from the Pea *(Pisum Sativum).* J. Biol. Chem. 1908–09, **5**, 197–205.

493. Osborne, T. B. and Heyl, F. W.: Hydrolysis of Fish Muscle. Amer. J. Physiol. 1908–09, **23**, 81–89.

494. Osborne, T. B., Leavenworth, C. S. and Brautlecht, C. A.: The Different Forms of Nitrogen in Proteins. Amer. J. Physiol. 1908–09, **23**, 180–200.

495. Osborne, T. B. and Jones, D. B.: Hydrolysis of Vitellin from Hen's Egg. Amer. J. Physiol. 1909, **24**, 153–160.

496. Osborne, T. B. and Jones, D. B.: Hydrolysis of the Muscle of Scallop *(Pectens Iradians).* Amer. J. Physiol. 1909, **24**, 161–169.

497. Osborne, T. B., Jones, D. B. and Leavenworth, C. S.: Hydrolysis of Crystallized Albumin from Hen's Egg. Amer. J. Physiol. 1909, **24**, 252–262.

498. Osborne, T. B. and Jones, D. B.: Hydrolysis of Ox Muscle. Amer. J. Physiol. 1909, **24**, 437–446.

499. Osborne, T. B. and Jones, D. B.: Some Modifications of the Method in Determining the Quantity of Mono-Amino-Acids Yielded by Proteins when Hydrolyzed with Acids. Amer. J. Physiol. 1910, **26**, 212–228.

500. Osborne, T. B. and Liddle, L. M.: Notes on the Analysis of Edestin and Zein. Amer. J. Physiol. 1910, **26**, 295–304.

501. Osborne, T. B. and Jones, D. B.: A Consideration of the Sources

of Loss in Analyzing the Products of Protein Hydrolysis. Amer.
J. Physiol. 1910, **26**, 305–328.

502. Osborne, T. B. and Guest, H. H.: Hydrolysis of Casein. J. Biol.
Chem. 1911, **9**, 333–353.

503. Osborne, T. B. and Guest, H. H.: Analysis of the Products of Hy-
drolysis of Wheat Gliadin. J. Biol. Chem. 1911, **9**, 425–438.

504. Osborne, T. B. and Mendel, L. B.: Amino-Acids in Nutrition and
Growth. J. Biol. Chem. 1914, **17**, 325–349.

505. Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S. and Vino-
grad, M.: Some Products of Hydrolysis of Gliadin, Lactalbumin,
and the Protein of the Rice Kernel. J. Biol. Chem. 1915, **22**, 259–
280.

506. Osborne, T. B., Leavenworth, C. S. and Nolan, L. S.: A Note on
Dakin's Method as Applied to Edestin. J. Biol. Chem. 1924, **61**,
309–313.

507. Osborne, T. B.: The Vegetable Proteins. Longmans, Green and Co.,
New York, 1924, 154 pages.

508. Padoa, M. L.: The Amino Acid Content of Glutin and its Food
Value. Ann. chim. applicata 1938, **27**, 544–554, from Chem. Abs.
1938, **32**, 6297.

509. Painter, E. P. and Franke, K. W.: A Comparison between the
Benedict-Denis and Parr Bomb Methods for the Determination of
Total Sulfur in Plants and Proteins. J. Biol. Chem. 1936, **114**,
235–239.

510. Palmer, J. W., Smyth, E. M. and Meyer, K.: On Glycoproteins. IV.
The Estimation of Hexosamine. J. Biol. Chem. 1937, **119**, 491–500.

511. Patton, A. R.: The Determination of Glycine in Proteins. J. Biol.
Chem. 1935, **108**, 267–272.

512. Patton, A. R. and Palmer, L. S.: The Amino Acid Content of Eggs
and Chicks: Relation to Diet and to Incidence of Chondrodys-
trophy. J. Nutr. 1936, **11**, 129–134.

513. Pauly, H.: Über die Konstitution des Histidins. Zeit. physiol.
Chem. 1904, **42**, 508–518.

514. Pauly, H.: Zur Kenntnis der Diazoreaktion des Eiweisses. Zeit.
physiol. Chem. 1915, **94**, 284–290.

515. Pauly, H. and Ludwig, E.: Glucosamin als Bildner heterocyclischer
Verbindungen. Zeit. physiol. Chem. 1922, **121**, 170–176.

516. Peters, J. B. and Van Slyke, D. D.: Quantitative Clinical Chemistry
Vol. II. Williams and Wilkins Co., Baltimore, Md. 1932, 616
pages.

517. Phelps, I. K. and Phelps, M. A.: The Use of Zinc Chloride in the
Esterification of Succinic Acid. Amer. J. Science 1907, **24**, 194–196.

518. Plimmer, R. H. A.: The Analysis of Proteins. I. The Estimation of
Arginine by Decomposition with Alkali. Biochem. J. 1916, **10**,
115–119.

519. Plimmer, R. H. A. and Phillips, H.: The Analysis of Proteins. III.

Estimation of Histidine and Tyrosine by Bromination. Biochem. J. 1924, 18, 312–321.

520. Plimmer, R. H. A. and Rosedale, J. L.: Analysis of Proteins. V. Van Slyke's Method of Determination of Nitrogen Distribution. Biochem. J. 1925, 19, 1004–1014.

521. Plimmer, R. H. A. and Lowndes, J.: Analysis of Proteins. IX. The Content in Amino-Acids of the Caseinogen and Lactalbumin of Woman's Milk. Biochem. J. 1937, 31, 1751–1757.

522. Plimmer, R. H. A. and Lowndes, J.: Analysis of Proteins. X. Further Examination of Van Slyke's Method, and Estimation of Tryptophane by Bromination. Comp. rend. Lab. Carlsberg 1938, 22, 434–440.

523. Plimmer, R. H. A. and Lawton, J. H. T.: Analysis of Proteins. XI. The Products of the Action of Sodium Hydroxide on Caseinogen. The Composition of Dephosphocasein or Depocasein. Biochem. J. 1939, 33, 530–542.

524. Pollard, A. and Chibnall, A. C.: The Proteins of Grasses. III. The Cystine Content of Certain Grasses and other Pasture Plant Proteins. Biochem. J. 1934, 28, 326–336.

525. Pope, C. G. and Stevens, M. F.: The Determination of Amino Nitrogen Using a Copper Method. Biochem. J. 1939, 33, 1070–1077.

526. Pottinger, S. R.: Harrison, R. W. and Anderson, A. W.: Effect of Method of Manufacture on the Composition of Haddock Fish-Meal Proteins. U. S. Dept. of Commerce, Investigational Rept. #31, 1935.

527. Pottinger, S. R. and Baldwin, W. H.: The Content of Certain Amino Acids in the Edible Portions of Fishery Products. Proc. Sixth Pacific Sci. Congr. 1939, pages 453–459.

528. Potts, A. M. and Gallagher, T. F.: Cystine, Tyrosine, and Arginine Content of High Potency Pressor and Oxytocic Pituitary Hormones. J. Biol. Chem. 1942. 143, 561–562.

529. Pregl, F.: Über die Eihaute von *Scyllium Stellare* Günth und ihre Abbauproducte. Zeit. physiol. Chem. 1908, 56, 1–10.

530. Prunty, F. T. G.: The Determination of Cystine in Biological Material. Biochem. J. 1933, 27, 387–390.

531. von Przylecki, St. J. and Kasprzyk, K.: Eine neue Methode zur Trennung der Aminosäuren. Biochem. Zeit. 1936–37, 289, 242–250.

532. Pucher, G. W., Vickery, H. B. and Wakeman, A. J.: Determination of Malic Acid in Plant Tissue. Simultaneous Determination of Citric and Malic Acids. Ind. and Eng. Chem., Anal. Ed. 1934, 6, 288–291.

533. Pucher, G. W., Sherman, C. C. and Vickery, H. B.: A Method to Determine Small Amounts of Citric Acid in Biological Material. J. Biol. Chem. 1936, 113, 235–245.

534. Pucher, G. W. and Vickery, H. B.: A Method for Determining Glutamine in Plant Tissue. Ind. and Eng. Chem., Anal. Ed. 1940, 12, 27–29.

535. Pucher, G. W. and Vickery, H. B.: Determination of Succinic Acid in Plant Tissues. Ind. and Eng. Chem., Anal. Ed. 1941, 13, 412–415.

536. Racker, E.: Histidine Detection and Estimation in Urine. Biochem. J. 1940, 34, 89–96.

537. Rapoport, S.: Über eine Bestimmung der Summe von Glykokoll und Serin. Biochem. Zeit. 1935, 281, 30–36.

538. Rapoport, S. and Eichinger, W.: Beiträge zur Tryptophanreaktion nach Voisenet. Biochem. Zeit. 1936–37, 289, 288–289.

539. Rapoport, S.: Über die Bestimmung der Glycerinsäure in freier und veresterter Form. Biochem. Zeit. 1936–37, 289, 406–410.

540. Ratner, S. and Clarke, H. T.: The Action of Formaldehyde upon Cysteine. J. Amer. Chem. Soc. 1937, 59, 200–206.

541. Reach, F.: Quantitative Untersuchungen über das Tyrosin als Spaltungsproduct der Eiweisskörper. Virchow's Arch. 1899, 158, 288–296.

542. Rees, H. G.: The Amino Acids of the Mixed Proteins of Ox-Muscle; The Basic Amino Acids. Analyst. 1936, 61, 160–164.

543. Reiner, M. and Sobotka, H.: Tyrosine and Cystine Content of Serum Proteins. J. Biol. Chem. 1933, 100, 779–781.

544. Reiter, C.: Tyrosine Determinations. Science 1938, 88, 379.

545. Rhode, E.: Die Farbenreaktionen der Eiweisskörper mit p-Dimethyl-aminobenzaldehyde und anderer aromatischen Aldehyden. Zeit. physiol. Chem. 1905, 44, 161–170.

546. Rimington, C.: The Colorimetric Determination of Cystine by Means of the Uric Acid Reagent. Biochem. J. 1930, 24, 1114–1118.

547. Rittenberg, D. and Foster, G. L.: A New Procedure for Quantitative Analysis by Isotope Dilution, with Application to the Determination of Amino Acids and Fatty Acids. J. Biol. Chem. 1940, 133, 737–744.

548. Roche, A.: Recherches sur la constitution des proteines musculaires. III. Composition des proteines musculaires chez l'animal mort par inanition protéique et réserves azotées musculaires. Bull. Soc. Chim. Biol. 1934, 16, 270–284.

549. Roche, J. and Jean, G.: Sur la Composition en Acides Aminés des Hemocyanines et d'une Hémerythrine. Comp. rend. Soc. Biol. 1934, 115, 1645–1646.

550. Roche, J.: Essai sur la biochemie générale et comparée des pigments respiratoires. Masson et Cie, Paris 1936, 170 pages.

551. Roche, J. and Blanc-Jean, G.: Sur l'etat des groupments guanidiques dans les molécules protéiques. Comp. rend. Acad. Sciences 1940, 210, 681–683, from Chem. Abs. 1940, 34, 5102.

552. Roche, J. and Mourgue, M.: Globins. IV Leucine, Valine, and Alanine Contents and Specificity of Respiratory Pigments. (Blood and Muscle Hemoglobins, Erythrocruorins and Hemocyanins.) Trav. Membres Soc. chim. Biol. 1941, 23, 1329–1338, from Chem. Abs. 1942, 36, 7036.

553. Rosedale, J. L. and Da Silva, G. A.: The Amino Acids of Tissues. VI.

Determination of the Basic Amino-Acids in Small Quantities of Protein. Biochem. J. 1932, **26**, 369–376.

554. Rosner, L.: The Reaction between Iodoacetic Acid and Denatured Egg Albumin. J. Biol. Chem. 1940, **132**, 657–662.

555. Ross, A. F.: The Sulfur Distribution in Tobacco Mosaic Virus Protein. J. Biol. Chem. 1940, **136**, 119–129.

556. Ross, A. F.: The Determination of Some Amino Acids in Tobacco Mosaic Virus Protein. J. Biol. Chem. 1941, **138**, 741–749.

557. Ross, A. F.: The Fractionation of the Amino Acids of Tobacco Mosaic Virus Protein. J. Biol. Chem. 1942, **143**, 685–693.

558. Rossouw, S. D. and Wilken-Jorden, T. J.: Studies on the Origin of Sulphur in Wool. II. A Cuprous Mercaptide Method for the Determination of Cystine and Cysteine. Biochem. J. 1935, **29**, 219–224.

559. Ruhemann, S.: Triketohydrindene Hydrate. III. Its Relation to Alloxan. J. Chem. Soc. (Transactions) 1911, **99**, 792–800, 1486–1492.

560. Rutherford, H. A. and Harris, M. in Steinhardt, J. and Harris, M.: Combination of Wool Protein with Acid and Base: Hydrochloric Acid and Potassium Hydroxide. J. Research N.B.S. 1940, **24**, 335–367.

561. Rutherford, H. A., Harris, M. and Smith, A. L.: Estimation of Amino Nitrogen in Insoluble Proteins. J. Research N.B.S. 1937, **19**, 467–477.

562. Sakaguchi, S.: Über eine neue Farbenreaktion von Protein und Arginin. J. Biochem. (Japan) 1925, **5**, 25–31.

563. Sakaguchi, S.: Über die Bindungsweise und Quantitative Bestimmung des Arginins im Proteinmolecule. J. Biochem. (Japan) 1925, **5**, 133–142.

564. Sandstrom, W. M. and Lillivik, H. A.: Preparation of a Solution of o-Phthalaldehyde for Use as a Glycine Reagent. Ind. and Eng. Chem., Anal. Ed. 1941, **13**, 781.

565. Sato, M., Hirano, T. and Kan, T.: Okuda's Iodine Method for Determination of Cystine. Bull. Agr. Chem. Soc. (Japan) 1939, **15**, 123–124, from Chem. Abs. 1940, **34**, 3776.

566. Schild, E. and Enders, C.: Zur Spezifität des Phenolreagens nach Folin und Ciocalteu für die Tryptophanbestimmung. Biochem. Zeit. 1936, **286**, 220–224.

567. Schoenheimer, R., Ratner, S. and Rittenberg, D.: Studies in Protein Metabolism. X. The Metabolic Activity of Body Proteins Investigated with $l(-)$Leucine Containing Two Isotopes. J. Biol. Chem. 1939, **130**, 703–732.

568. Schöberl, A. and Rambacher, P.: Über die kolorimetrische Bestimmung von Cystein und Cystin mit Phosphorwolframsäure. Biochem. Zeit. 1937–38, **295**, 377–390.

569. Schmidt, C. L. A.: The Chemistry of the Amino Acids and Proteins. Charles C Thomas, Springfield, Ill. 1938, 1031 pages.

570. Schryver, S. B., Buston, H. W. and Mukherjee, D. H.: The Isolation of a Product of Hydrolysis of the Proteins Hitherto Undescribed. Proc. Roy. Soc. London, Ser. B. 1925, **98**, 58–65.

571. Schryver, S. B. and Buston, H. W.: The Isolation of Some Hitherto Undescribed Products of Hydrolysis of Proteins. Proc. Roy. Soc. London, Ser. B. 1926, **99**, 476–478.

572. Schultz, J. and Vars, H. M.: Note on the Graff Modification of the Vickery-White Procedure for the Estimation of Cystine. Proc. Soc. Biol. Chem.; J. Biol. Chem. 1941, **140**, cxiii–cxiv.

573. Schulz, F. N.: Die Bindungsweise des Schwefels im Eiweiss. Zeit. physiol. Chem. 1898, **25**, 16–35.

574. Schulze, E., Barbieri, J. and Bosshard, E.: Untersuchungen über die Amidosäuren, welche bei der Zersetzung der Eiweisstoffe durch Salzsäure und durch Barytwasser entstehen. Zeit. physiol. Chem. 1885, **9**, 63–126.

575. Sharp, J. G.: The Amino Acid Composition of Rabbit Myosin. Biochem. J. 1939, **33**, 679–693.

576. Shaw, J. L. D. and McFarlane, W. D.: The Determination of Tryptophane by a Modified Glyoxylic Acid Method Employing Photoelectric Colorimetry. Canad. J. Research 1938, **16B**, 361–368.

577. Shaw, J. L. D. and McFarlane, W. D.: A Critical Study of the p-Dimethylaminobenzaldehyde Method for Determining Tryptophane as Contrasted with the Glyoxylic Acid Method. J. Biol. Chem. 1940, **132**, 387–392.

578. Shinn, L. A. and Nicolet, B. H.: The Determination of Threonine by the Use of Periodate. J. Biol. Chem. 1941, **138**, 91–96.

579. Shinohara, K. and Kilpatrick, M.: The Stability of Cystine in Acid Solution. J. Biol. Chem. 1934, **105**, 241–251.

580. Shinohara, K.: The Determination of Thiol and Disulfide Compounds, with Special Reference to Cysteine and Cystine. I. Critical Study of the Color Reaction between Phospho-18-Tungstic Acid Reagent and Thiol Compounds. J. Biol. Chem. 1935, **109**, 665–679.

581. Shinohara, K.: The Determination of Thiol and Disulfide Compounds, with Special Reference to Cysteine and Cystine. II. A Critical Study of the Reaction between Phospho-18-Tungstic Acid and Other Substances than Thiol Compounds. J. Biol. Chem. 1935, **110**, 263–277.

582. Shinohara, K.: The Determination of Thiol and Disulfide Compounds, with Special Reference to Cysteine and Cystine. V. A Critical Study of Cystine Determination of Sulfite and Phospho-18-Tungstic Acid Reagent. J. Biol. Chem. 1935, **112**, 683–696.

582A. Snell, E. E. and Wright, L. D.: A Microbiological Method for the Determination of Nicotinic Acid. J. Biol. Chem. 1941, **139**, 675–686.

583. Sørensen, M.: On the Determination of Glucosamine in Proteins. Comp. rend. Lab. Carlsberg 1938, **22**, 487–493.

584. Speakman, J. B. and *Townend, F.*: Constitution of the Keratin Molecule. Nature 1937, **139**, 411–412.

585. Spies, J. R.: The Chemistry of Allergens. V. The Amino Acid Content of Active Protein and Polysaccharidic Fractions from Cottonseed. J. Amer. Chem. Soc. 1941, **63**, 2994–2996.

586. Stein, W. H. and Miller, E. G., Jr.: The Composition of Elastin. J. Biol. Chem. 1938, **125**, 599–614.

587. Stein, W. H. and Bergmann, M.: Determination of Proline in Mixtures Containing *l* and *dl*-Proline. The Proline Content of Gelatin. J. Biol. Chem. 1940, **134**, 627–633.

588. Stein, W. H., Moore, S., Stamm, G., Chou, C. and Bergmann, M.: Aromatic Sulfonic Acids as Reagents for Amino Acids. The Preparation of *l*-Serine, *l*-Alanine, *l*-Phenylalanine, and *l*-Leucine from Protein Hydrolysates. J. Biol. Chem. 1942, **143**, 121–129.

588A. Stein, W. H.: Some Current Problems and Recent Advances in the Chemistry of the Proteins. Trans. Amer. Soc. Cereal Chem. 1943, **57**, 11–25.

589. Steinhardt, J. and Fugitt, C. H.: Catalyzed Hydrolysis of Amide and Peptide Bonds in Proteins. J. Research N.B.S. 1942, **29**, 315–327.

590. Stern, A., Beach, E. F. and Macy, I. G.: Polarographic Microdetermination of Cystine in Protein Hydrolysates. J. Biol. Chem. 1939, **130**, 733–740.

591. Stern, A. and Beach, E. F.: Microdetermination of Homocystine in Pure Solution with the Dropping Mercury Cathode. Proc. Soc. Exp. Biol. and Med. 1940, **43**, 104–108.

592. Steudel, H.: Isolierung, Bestimmung und Nachweis von Histidin, Lysin, und Arginin (Ornithin und Guanidin). Biochem. Arbeitsmethoden (E. Abderhalden) 1923, Sect. I, Part 7, 203–224.

593. Steudel, H. and Osato, S.: Über die Zusammensetzung der Heringseier. II. Die Eischalen. Zeit. physiol. Chem. 1923, **127**, 220–223.

594. Strain, H. H.: Chromatographic Adsorption Analysis. Interscience Publishers, New York 1942, 222 pages.

595. Sullivan, M.X.: A Distinctive Test for Cysteine. Pub. Health Reps.; U.S.P.H.S. 1926, **41**, 1030–1056.

596. Sullivan, M. X. and Hess, W. C.: Studies on the Biochemistry of Sulphur. VII. The Cystine Content of Purified Proteins. Pub. Health Reps.; U.S.P.H.S. 1930, supp. 86, 1–11.

597. Sullivan, M. X. and Hess, W. C.: The Determination of Cystine in Urine. J. Biol. Chem. 1936, **116**, 221–232.

598. Sullivan, M. X. and Hess, W. C.: Improvements in Methods of Hydrolysis of Protein: Shortening the Time for Estimating Cystine. J. Biol. Chem. 1937, **117**, 423–428.

599. Sullivan, M. X., Milone, H. S. and Everitt, E. L.: A Rapid Procedure for Estimating the Tryptophane Content of Casein. J. Biol. Chem. 1938, **125**, 471–474.

600. Sullivan, M. X., Hess, W. C. and Smith, F. R.: A Comparison of Colorimetric and Polarographic Methods in Relation to the Cystine Content of Insulin. J. Biol. Chem. 1939, **130**, 741–744.

601. Sullivan, M. X., Hess, W. C. and Howe, P. E.: Comparison of the Wool and Skins of Full-Fed and Maintenance-Fed Lambs. J. Agri. Research 1940, 61, 877–886.

602. Sullivan, M. X., Hess, W. C. and Howard, H. W.: The Quantitative Estimation of both Cystine and Cysteine in Mixtures. J. Biol. Chem. 1942, 145, 621–624.

603. Teruuchi, Y. and Okabe, L.: On a Modified Method of Okuda's Cystine Estimation and the Cystine Content of Several Kinds of Proteins. J. Biochem. (Japan) 1928, 8, 459–467.

604. Theis, E. R. and Jacoby, T. F.: The Acid-Base-Binding Capacity of Collagen. J. Biol. Chem. 1942, 146, 163–169.

605. Theorell, H. and Åkesson, Å.: Studies on Cytochrome C. I. Electrophoretic Purification of Cytochrome C and its Amino Acid Composition. J. Amer. Chem. Soc. 1941, 63, 1804–1811.

606. Thimann, K. V.: The Precipitation of the Basic Amino-Acids of Proteins with Phosphotungstic Acid. Biochem. J. 1930, 24, 368–378.

607. Thomas, L. E., Ingalls, J. K. and Luck, J. M.: The Determination of Arginine in the Presence of other Amino Acids by Means of the Sakaguchi Reaction. J. Biol. Chem. 1939, 129, 268–271.

608. Thomas, P.: Sur le Dosage du Tryptophane dans les Matières Protéiques. Ann. de l'Inst. Pasteur 1920, 34, 701–708.

608A. Thompson, W. T.: Microbiological Assays of the Amino Acids. Chem. and Eng. News 1943, 21, 1662.

609. Tiselius, A.: A New Procedure for Adsorption Analysis. Science 1941, 94, 145–146.

610. Tiselius, A.: Adsorption Analysis of Amino Acids and Peptides. Ark. Kemi, Minerol. o. Geol. 1941, 15B, 1–5.

611. Tiselius, A, in Kraemer, E. O.; Advances in Colloid Science. Interscience Publishing Co., New York 1942, 434 pages.

612. Toennies, G.: The Sulfur Containing Amino Acid Methionine. Growth. 1937, 1, 337–370.

613. Toennies, G.: The Oxidative Conversion of Casein into Protein Free of Methionine and Tryptophane. J. Biol. Chem. 1942, 145, 667–670.

614. Tomiyama, T. and Shigematsu, S.: A Modified Method for the Estimation of Tryptophane. Proc. Soc. Exp. Biol. and Med. 1934–35, 32, 446–449.

615. Tompsett, S. L.: A Note on the Determination of Cystine in Proteins by the Method of Folin and Marenzi. Biochem. J. 1931, 25, 2014–2016.

616. Town, B. W.: The Isolation of Pure l-Proline. Biochem. J. 1928, 22, 1083–1086.

617. Town, B. W.: The Micro-Determination of Glycine in Protein Hydrolysates. Biochem. J. 1936, 30, 1833–1836.

618. Toyoda, H.: A Micro-Method for the Determination of Disulfide and Sulphydryl-Compounds. I. Bull. Chem. Soc. (Japan) 1934, 9, 263–268.

619. Tristram, G. R.: The Basic Amino-Acids of Leaf Proteins. With a Discussion of Various Methods of Analysis. Biochem. J. 1939, **33,** 1271–1283.

620. Tristram, G. R.: The Proteins 'of *Hevea Brasiliensis*. I. Analysis of a Product Isolated from Dried Latex. Biochem. J. 1940, **34,** 301–306.

621. Tuchman, L. R. and Reiner, M.: The Cystine Content of Human Serum Proteins. J. Biol. Chem. 1933, **100,** 775–778.

622. Turba, F.: The Adsorption Behavior of Protein-Degradation Products. I. Chromatographic Adsorption of Basic Amino Acids on Bleaching Earths. Ber. deut. chem. Gesel. 1941, **74B,** 1829–1838, from Chem. Abs. 1942, **36,** 5494.

623. Tutiya, Y.: Methionine and its Derivatives. I. Detection. J. Agr. Chem. Soc. (Japan) 1941, **17,** 48–49, from Chem. Abs. 1942, **36,** 3752.

624. Urbach, C.: Quantitative Bestimmung des Acetons im Harn. Mittels des Stufenphotometers. Biochem. Zeit. 1931, **236,** 164–173.

625. Urban, F. F.: Zur Kenntnis des chemischen Aufbaues von Leberproteinen. Biochem. Zeit. 1936, **283,** 435–453.

626. Ussing, H. H.: Analysis of Protein by Means of Deuterium-containing Amino-Acids. Nature 1939, **144,** 977.

627. Van Slyke, D. D.: Eine Methode zur quantitativen Bestimmung der Aliphatischen Amino gruppen; einige Anwendung derselben in der Chemie der Proteine, des Harns, und der Enzyme. Ber. deut. chem. Gesel. 1910, **43,** 3170–3181.

628. Van Slyke, D. D.: A Method for Quantitative Determination of Aliphatic Amino Group. Applications to the Study of Proteolysis and Proteolytic Products. J. Biol. Chem. 1911, **9,** 185–204.

629. Van Slyke, D. D.: Quantitative Determination of Prolin Obtained by the Ester Method in Protein Hydrolysis. Prolin Content of Casein. J. Biol. Chem. 1911, **9,** 205–207.

630. Van Slyke, D. D.: The Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino-Acids. J. Biol. Chem. 1911–12, **10,** 15–55.

631. Van Slyke, D. D.: The Hexone Bases in Casein. J. Biol. Chem. 1913–14, **16,** 531–538.

632. Van Slyke, D. D. and Birchard, F. J.: The Nature of the Free Amino Groups in Proteins. J. Biol. Chem. 1913–14, **16,** 539–547.

633. Van Slyke, D. D.: Improvements in the Method for Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino-Acids. J. Biol. Chem. 1915, **22,** 281–285.

634. Van Slyke, D. D.: Correction Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino Acids. J. Biol. Chem. 1915, **23,** 411.

635. Van Slyke, D. D. and Hawkins, J. A.: A Gasometric Method for Determination of Reducing Sugars, and Its Application to Analysis of Blood and Urine. J. Biol. Chem. 1928, **79,** 739–767.

636. Van Slyke, D. D. and Dillon, R. T.: Gasometric Determination of Carboxyl Groups in Amino Acids. Comp. rend. Lab. Carlsberg. 1938, **22**, 480–486.

637. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. and Hamilton, P.: Gasometric Determination of Carboxyl Groups in Free Amino Acids. J. Biol. Chem. 1941, **141**, 627–669.

638. Van Slyke, D. D., MacFadyen, D. A. and Hamilton, P.: Determination of Free Amino Acids by Titration of the Carbon Dioxide Formed in the Reaction with Ninhydrin. J. Biol. Chem. 1941, **141**, 671–680.

639. Van Slyke, D. D., Hiller, A. and MacFadyen, D. A.: The Determination of Hydroxylysine in Proteins. J. Biol. Chem. 1941, **141**, 681–705.

640. Van Slyke, D. D., Hiller, A. and Dillon, R. T.: Solubilities and Compositions of the Phospho-12-Tungstates of the Diamino Acids and of Proline, Glycine, and Tryptophane. J. Biol. Chem. 1942, **146**, 137–157.

641. Vassel, B.: A Colorimetric Micromethod for the Estimation of Cystine and Cysteine. J. Biol. Chem. 1941, **140**, 323–336.

642. Vickery, H. B. and Leavenworth, C. S.: On the Separation of Histidine and Arginine. II. The Separation of the Silver Compounds at p_H 7.0. J. Biol. Chem. 1927, **72**, 403–413.

643. Vickery, H. B. and Leavenworth, C. S.: Modifications of the Method for the Determination of the Basic Amino Acids of Proteins. The Bases of Edestin. J. Biol. Chem. 1928, **86**, 707–722.

644. Vickery, H. B. and Leavenworth, C. S.: On the Separation of Histidine and Arginine. IV. The Preparation of Histidine. J. Biol. Chem. 1928, **78**, 627–635.

645. Vickery, H. B. and Leavenworth, C. S.: The Basic Amino Acids of Horse Hemoglobin. J. Biol. Chem. 1928, **79**, 377–388.

647. Vickery, H. B. and Leavenworth, C. S.: The Separation of Cystine from Histidine: The Basic Amino Acids of Human Hair. J. Biol. Chem. 1929, **83**, 523–534.

648. Vickery, H. B. and Block, R. J.: The Basic Amino Acids in Wool. J. Biol. Chem. 1930, **86**, 107–111.

649. Vickery, H. B. and Schmidt, C. L. A.: The History of the Discovery of the Amino Acids. Chem. Rev. 1931, **9**, 169–318.

650. Vickery, H. B. and Block, R. J.: The Basic Amino Acids of Silk Fibroin. The Determination of the Basic Amino Acids Yielded by Proteins. J. Biol. Chem. 1931, **93**, 105–112.

651. Vickery, H. B. and Shore, A.: The Basic Amino Acids of Crystalline Egg-Albumin. Biochem. J. 1932, **26**, 1101–1106.

652. Vickery, H. B. and White, A.: The Use of Cysteine Cuprous Mercaptide in the Determination of Cystine. J. Biol. Chem. 1932–33, **99**, 701–715.

653. Vickery, H. B. and White, A.: Proportion of Cystine Yielded by

Hemoglobins of the Horse, Dog, and Sheep. Proc. Soc. Exp. Biol. and Med. 1933–34, **31**, 6–7.

654. Vickery, H. B. and White, A.: The Basic Amino Acids of Casein. J. Biol. Chem. 1933, **103**, 413–415.

655. Vickery, H. B.: The Amino Acid Composition of Zein. Comp. rend. Lab. Carlsberg. 1938, **22**, 519–527.

656. Vickery, H. B.: The Determination of Arginine by Means of Flavianic Acid. J. Biol. Chem. 1940, **132**, 325–342.

657. Vickery, H. B., Smith, E. L., Hubbell, R. B. and Nolan, L. S.: Cucurbit Seed Globulins. I. Amino Acid Composition and Preliminary Tests of Nutritive Value. J. Biol. Chem. 1941, **140**, 613–624.

658. Vickery, H. B.: The Preparation of Histidine by Means of 3,4-Dichlorobenzenesulfonic Acid. J. Biol. Chem. 1942, **143**, 77–87.

659. Vickery, H. B.; The Histidine Content of the Hemoglobin of Man and of the Horse and Sheep. Determined with the Aid of 3,4 Dichlorobenzenesulfonic Acid. J. Biol. Chem. 1942, **144**, 719–730.

660. du Vigneaud, V., Jensen, H. and Wintersteiner, O.: Studies on Crystalline Insulin. III. Further Observations on the Crystallization of Insulin and on the Nature of the Sulfur Linkage. The Isolation of Cystine and Tyrosine from Hydrolyzed Crystalline Insulin. J. Pharm. and Exp. Therapeutics. 1927–28, **32**, 367–385.

661. du Vigneaud, V., Miller, G. L. and Rodden, C. J.: On the Question of the Presence of Methionine in Insulin. J. Biol. Chem. 1939, **131**, 631–640.

662. Virtanen, A. I. and Laine, T.: Ninhydrine Reaction in the Quantitative Determination of Different Amino Acids. Nature 1938, **142**, 754.

663. Virtanen, A. I., Laine, T. and Toivonen, T.: Quantitative Bestimmung von gewissen Aminosäuren nach der Ninhydrinmethode. Zeit. physiol. Chem. 1940, **266**, 193–204.

664. Virtue, R. W. and Lewis, H. B.: The Iodometric Determination of Cystine in the Urine. J. Biol. Chem. 1934, **104**, 415–421.

665. Voisenet, M. E.: Sur une réaction très sensible de la formaldéhyde et des composés oxygénés de l'azote et qui est aussi une réaction de coloration des matières albuminoides. Bull. Soc. Chim. 1905, **33**, 1198–1214.

666. Vorländer, D.: Methon als Aldehydreagens. Zeit. anal. Chem. 1929, **77**, 241–268.

667. Wachtel, J. L. and Cassidy, H. G.: Chromatography as a Means of Separating Amino Acids. J. Amer. Chem. Soc. 1943, **65**, 665–668.

668. Waelsch, H. and Klepetar, G.: Gravimetrische Mikrobestimmungen des Schwefels in biologischem Material. Zeit. physiol. Chem. 1932. **211**, 47–56.

669. Wakamatu, S.: Muscle Protein of Herring. J. Agri. Chem. Soc. (Japan) 1939, **15**, 915–920, from Chem. Abs. 1940, **34**, 1367.

670. Wakeman, A. J.: Über die Verteilung des Stickstoffs in der Leber des Störs. Zeit. physiol. Chem. 1905, **44, 341.**

671. Waldschmidt-Leitz, E. and Akabori, S.: Bestimmung von Oxy-prolin zur Methode nach K. Lang. Zeit. physiol. Chem. 1934, **224,** 187–192.

672. Webb, M. and Block, R. J.: Unpublished Experiments.

673. Weber, C. J.: A Modification of Sakaguchi's Reaction for the Quantitative Determination of Arginine. J. Biol. Chem. 1930, **86,** 217–222.

674. Weiss, M. and Ssobolew, N.: Über ein colorimetrisches Verfahren zur quantitativen Bestimmung des Histidins. Biochem. Zeit. 1913–14, **58,** 119–129.

675. Weiss, M.: Über den quantitativen Nachweis des Tyrosins mittels der Millonschen Reaction. Biochem. Zeit. 1919, **97,** 170–175.

676. Whitehorn, J. C.: "Permutit" as a Reagent for Amines. J. Biol. Chem. 1923, **56,** 751–764.

678. Wilkerson, V. A.: The Chemistry of Human Epidermis. I. Amino Acid Content of the Stratum Corneum and its Comparison to other Human Keratins. J. Biol. Chem. 1934, **107,** 37–38.

679. Wilkerson, V. A. and Tulane, V. J.: The Chemistry of Human Skin. III. The Occurrence of Methionine in Human Skin (Stratum Corneum). J. Biol. Chem. 1939, **129,** 477–479.

680. Wilson, H. and Cannan, R. K.: The Glutamic Acid-Pyrrolidone Carboxylic Acid System. J. Biol. Chem. 1937, **119,** 309–331.

681. Wilson, R. H. and Lewis, H. B.: The Cystine Content of Hair and Other Epidermal Tissues. J. Biol. Chem. 1927, **73,** 543–553.

682. Winkler, S.: Über die Tryptophanreaktion von Adamkiewicz-Hopkins. Zeit. physiol. Chem. 1934, **228,** 50–60.

683. Winnick, T.: Microdiffusion Methods Based on the Bisulfite Reaction. I. The Determination of Acetone. J. Biol. Chem. 1941, **141,** 115–120.

684. Winnick, T.: Microdiffusion Methods Based on the Bisulfite Reaction. II. Determination of Lactic Acid by Oxidation with Ceric Sulfate. J. Biol. Chem. 1942, **142,** 451–459.

685. Winnick, T.: Microdiffusion Methods Based on the Bisulfite Reaction. III. Determination of Threonine by Oxidation with Periodate. J. Biol. Chem. 1942, **142,** 461–466.

686. Winterstein, E.: Ueber eine Methode zur Abscheidung der organischen Basen aus den Phosphorwolframsäureneiderschlägen und über das Verhalten des Cystins gegen Phosphorwolframsäure. Zeit. physiol. Chem. 1901–02, **34,** 153–156.

687. Woodward, G. E., Reinhart, F. E. and Dohan, J. S.: The Glutamic Acid of Malignant Tissue Proteins. J. Biol. Chem. 1941, **138,** 677–688.

688. Woodward, G. E. and Reinhart, F. E.: The Effect of pH on the Formation of Pyrrolidonecarboxylic Acid and Glutamic Acid

during Enzymatic Hydrolysis of Glutathione by Rat Kidney Extract. J. Biol. Chem. 1942, **145**, 471–480.

689. Woolley, D. W. and Peterson, W. H.: The Chemistry of Mold Tissue. XIII. Isolation of Some Monoaminocarboxy and some Mono-Aminodicarboxy Acids from *Aspergillus Sydowi*. J. Biol. Chem. 1937, **121**, 507–520.

690. Woolley, D. W.: Improved Extractor for Monoamino Acids. Ind. and Eng. Chem., Anal. Ed. 1937, **9**, 433.

691. Woolley, D. W. and Peterson, W. H.: Some Observations on the Kapeller-Adler Method for the Determination of Histidine. The Histidine Content of Yeast. J. Biol. Chem. 1937–38, **122**, 207–211.

692. Wretlind, K. A. J.: Color Reaction with Isobutyraldehyde for the Colorimetric Determination of Valine. Acta Physiol. Scand. 1942, **3**, 329–334, from Chem. Abs. 1942, **36**, 7047.

693. Wu, H.: Contribution to the Chemistry of Phosphomolybdic Acids, Phosphotungstic Acids, and Allied Substances. J. Biol. Chem. 1920, **43**, 189–220.

694. Young, E. G. and Inman, W. R.: The Protein of the Casing of Salmon Eggs. J. Biol. Chem. 1938, **124**, 189–193.

695. Zahnd, H. and Clarke, H. T.: Labile Sulfur in Proteins. J. Biol. Chem. 1933, **102**, 171–186.

696. Zelinsky, N., Annenkoff, A. and Kulikoff, J. Über ein einfaches und bequemes Verfahren zur Darstellung der freien Ester der Amino-säuren. Zeit. physiol. Chem. 1911, **73**, 459–470.

697. Zimmermann, W.: Über den Nachweis kleiner Mengen Glykokoll. Zeit. physiol. Chem. 1930, **189**, 4–6.

698. Zittle, C. A. and O'Dell, R. A.: The Determination of Cystine: The Use of Cuprous Oxide for Simultaneous Reduction and Precipitation of Cystine as the Cuprous Merceptide. J. Biol. Chem. 1941, **139**, 753–759.

699. Zuwerkalow, D.: Zur Frage der Tyrosinbestimmung mit Hilfe der Millonschen Reaktion. Zeit. physiol. Chem. 1927, **163**, 185–192.

700. Beveridge, J. M. R. and Lucas, C. C.: Amino Acids of Isinglass. J. Biol. Chem. 1944, **155**, 547–556.

701. Block, R. J. and Bolling, D.: A Note on the Amino Acids Yielded by Yeast, Sunflower Seed Meal and Sesame Seed after Hydrolysis of the Fat Free Tissue. Arch. Biochem. 1945, **6**, 277–279.

702. Block, R. J. and Bolling, D.: The Amino Acids Yielded by Various Yeasts after Hydrolysis of the Fat-free Material. Arch. Biochem. 1945, **7**, 313–321.

703. Christensen, H. N., Uzman, L. and Hegsted, D. M.: A Note on the Amino Acid Composition of Tyrocidine. J. Biol. Chem. 1945, **158**, 279–281.

704. Devlin, H. B. and Zittle, C. A.: A Nutritional Study of Human Globin in Rats. J. Biol. Chem. 1944, **156**, 393–400.

705. Foster, G. I.: Some Amino Acid Analyses of Hemoglobin and β-Lactoglobulin. J. Biol. Chem. 1945, **159**, 431–438.

706. Hegsted, D. M.: The Amino Acid Requirements of Lactobacillus Arabinosus 17-5. J. Biol. Chem. 1943, **152,** 193–200.
707. Hodson, A. Z. and Krueger, G. M.: Direct Estimation of Leucine and Arginine (Application to Milk and Certain Other Foodstuffs). Private Communication.
708. McMahan, J. R. and Snell, E. E.: The Microbiological Determination of Amino Acids. I. Valine and Arginine. J. Biol. Chem. 1943, **152,** 83–95.
709. Regnery, D. C.: A Leucineless Mutant Strain of Neurospora Crassa. J. Biol. Chem. 1944, **154,** 151–160.
710. Ryan, F. J. and Brand, E.: A Method for the Determination of Leucine in Protein Hydrolysates and in Foodstuffs by the Use of a Neurospora Mutant. J. Biol. Chem. 1944, **154,** 161–175.
711. Schweigert, B. S., Tatam, I. E. and Elvehjem, C. A.: The Leucine, Isoleucine, and Valine Content of Meats. Arch. Biochem. 1945, **6,** 177–184.
712. Williamson, M. B.: The Amino Acid Composition of Human Milk Proteins. J. Biol. Chem. 1944, **156,** 47–52.

AUTHOR INDEX

353

SUBJECT INDEX

A

362

This Book

The

Amino Acid Composition

of

Proteins and Foods

ANALYTICAL METHODS AND RESULTS